PROTEOMIC-BASED PROFILING OF THE LEAF EXTRACTS OF *FICUS DELTOIDEA*

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Abstract

Ficus deltoidea is one of the well-known herbs in Malay traditional medicine. This plant is commonly known as Mas Cotek in Malaysia. According to traditional medicinal practitioners, the decoction of the plant is able to prevent and cure various ailments. Recent pharmacological studies suggested that the aqueous extract contains pharmacologically active constituents that support the traditional use of this plant. The aim of this study is to investigate and compare the protein present in *F. deltoidea* var. *angustifolia* (SL), *F. deltoidea* var. *trengganuensis* (ML) and *F. deltoidea* var. *kunstleri* (BL) leaves aqueous extract using proteomic techniques. In sample preparation, protein precipitation is a crucial step in removing interfering compound that may affect the proteomic analysis and also to concentrate the proteins in the leaves aqueous extracts. Among the precipitation method evaluated, double precipitation method which consists of ammonium sulfate and acetone/β-mercaptoethanol precipitation was selected as the most suitable method to precipitate the protein out from the extracts as it gave higher protein purity and better band resolution on SDS-PAGE analysis. Samples obtained from this precipitation method were subjected to proteomic analysis and revealed a total of 45, 24 and 9 protein spots on the proteome of SL, ML and BL respectively. All of them are lower than 78 kDa. Proteins below 6 kDa were underrepresented on 2-D proteome profile due to the limitation of 2-D gel electrophoresis, but were revealed on SDS-PAGE proteome profile. SL proteome contains of a group of characteristic proteins in the range of 6 – 26 kDa that are not present in ML and BL proteome, whereas ML and BL have similar proteome. SELDI-TOF profiling also showed similarity in ML and BL spectrum profile with 4 signal peaks detected, while there are only 3 signal peaks obtained in SL SELDI-TOF profile. The missing signal peak at *m/z* 3825 had differentiated SL from ML and BL. Besides that, decoction of *Ficus* leaves also contains some glycoproteins which lead to the smearing background.

Abstrak

Ficus deltoidea adalah salah satu herba tradisional Melayu yang terkenal. Ia dikenali sebagai Mas Cotek di Malaysia. Menurut pengamal perubatan tradisional, air rebusan tumbuhan ini mampu mencegah dan menyembuhkan pelbagai penyakit. Kajian farmakologi mencadangkan bahawa air rebusan tumbuhan ini mengandungi bahan aktif yang menyokong penggunaan tradisional tumbuhan ini. Tujuan kajian ini adalah untuk menyiasat dan membandingkan protein yang hadir dalam ekstrak akues daun *F. deltoidea* var. *angustifolia* (SL), *F. deltoidea* var. *trengganuensis* (ML) dan *F. deltoidea* var. *kunstleri* (BL) dengan teknik proteomik. Pemendakan protein merupakan satu langkah yang penting untuk membuangkan bahan asing yang boleh menjejaskan analisa proteomik dan juga menepukan protein di dalam ekstrak daun. Kaedah pemendakan berganda yang menggabungkan pemendakan amonium sulfat dan aseton/β-mercaptoethanol telah dipilih sebagai kaedah yang paling sesuai untuk memendakan protein dari ekstrak *Ficus* kerana ia memberikan ketulenan protein yang tinggi dan resolusi yang baik bagi analisis SDS-PAGE. Analisis proteomik menunjukkan bilangan protein sebanyak 45, 24 dan 9 di dalam proteom SL, ML dan BL masing-masing, dengan berat molekul kurang daripada 78 kDa. Disebabkan terdapatnya batasan elektroforesis 2-D gel (2-DE), protein dengan berat molekul di bawah 6 kDa tidak dapat dilihat dalam profail proteom 2-DE, tetapi ditunjukkan dalam profail proteom SDS-PAGE. Proteom SL mengandungi sekumpulan protein dalam berat molekul 6 – 26 kDa yang tidak ada dalam proteom ML dan BL, manakala ML dan BL mempunyai proteom yang serupa. Analisis SELDI juga menunjukkan persamaan profail di antara ML dan BL dengan 4 puncak signal dikesan. Hanya ada 3 puncak signal didapati dalam profail SELDI SL. Signal puncak *m/z* 3825 yang hilang dalam profail SELDI SL membezakannya dengan ML dan BL. Selain itu, ekstrak *Ficus* juga mengandungi glikoprotein yang menyebabkan gangguan pada latar belakang proteom.

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List of Symbols and Abbreviations

Samples Abbreviation

F. deltoidea	=	*Ficus deltoidea*
SL	=	Small-type leaves OR *Ficus deltoidea* var. *angustifolia*
ML	=	Medium-type leaves OR *Ficus deltoidea* var. *trengganuuensis*
BL	=	Big-type leaves OR *Ficus deltoidea* var. *kunstleri*
SL1	=	SL extract obtained from ammonium sulfate precipitation
SL2	=	SL extract obtained from cold acetone precipitation
SL3	=	SL extract obtained from TCA/acetone cold precipitation
SL4	=	SL extract obtained from ammonium sulfate precipitation combined with acetone precipitation
ML1	=	ML extract obtained from ammonium sulfate precipitation
ML2	=	ML extract obtained from cold acetone precipitation
ML3	=	ML extract obtained from TCA/acetone cold precipitation
ML4	=	ML extract obtained from ammonium sulfate precipitation combined with acetone precipitation
BL1	=	BL extract obtained from ammonium sulfate precipitation
BL2	=	BL extract obtained from cold acetone precipitation
BL3	=	BL extract obtained from TCA/acetone cold precipitation
BL4	=	BL extract obtained from ammonium sulfate precipitation combined with acetone precipitation

Chemicals Abbreviation

APS	=	Ammonium persulfate
BSA	=	Bovine serum albumin
CHAPS	=	Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CHCA	=	α-Cyano-4-hydroxycinnamic acid
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminediacetic acid
HCl	=	Hydrochloric acid
PVPP	=	Polyvinylpyrrolidone
SDS	=	Sodium dodecyl sulfate
TCA	=	Trichloroacetic acid
TEMED	=	N,N,N’,N’-tetramethylethylenediamine
TFA	=	Trifluoroacetic acid

Units Abbreviation

%	=	Percentage
°C	=	Degree celcius
µA	=	Microampere
µg	=	Microgram
µL	=	Microlitre
cm	=	Centimeter
Da	=	Dalton
g	=	Gram
kDa	=	Kilo Dalton
L	=	Litre
M	=	Molar
m	=	Meter
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar

V	=	Volt
X	=	Times
Ppm	=	Part per million
W	=	Watt

Others

α	=	Alpha
β	=	Beta
>	=	More than
2^{nd}	=	Second
2-DE	=	Two-dimensional gel electrophoresis
2-D	=	Two-dimensional
DNA	=	Deoxyribonucleic acid
ESI	=	Electrospray ionization
FW	=	Formula weight
IEF	=	Isoelectric focusing
IPG	=	Immobilised pH gradient
i.e.	=	That is
Log	=	Logarithm
Log M_r	=	Logarithm of protein molecular mass
MALDI	=	Matrix assisted laser desorption/ionization
min	=	Minute
MS	=	Mass spectrometry
MW	=	Molecular weight
m/z	=	Mass-to-charge
P value	=	Probability value

pI	=	Isoelectric point
R_f	=	Relative mobility
rpm	=	Revolutions per minute
SDS-PAGE	=	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SELDI-TOF	=	Surface-Enhanced Laser Desorption/Ionization – time of flight
S/N	=	Signal to noise ratio
TOF	=	Time of flight
UV	=	Ultra violet
var.	=	Variety
v/v	=	Volume over volume
w/v	=	Weight over volume
WHO	=	World Health Organzation
x g	=	Times gravity

Chapter 1 : Introduction

1.1 Introduction

Herbs or medicinal plants have been playing a prominent role in ancient traditional medicine system and they have been a rich source of therapeutic agent for thousands of years. The pharmacological treatment of disease using herbs began long ago (Schulz *et al.*, 2001). Every tribe or race have their own methods of curing diseases and ailments and the knowledge of folk methods is imparted orally and comitted to memory. According to World Health Organzation (WHO), more than 80% of the population living in rural area are relying on traditional remedies for healthcare (Chronicle, 2013; Wilkinson, 2013). The use of herbal drugs is becoming more popular in the form of food supplement, nutraceuticals, and complementary and alternative medicine (Colegate *et al.*, 2007). In some developing countries, people are using it as an alternative to modern medicine (Robinson and Zhang, 2011). Increasing popularity of traditional herbs as health supplements and medical purposes has raise public concern on the safty and effectiveness of the herbs, and arouse the interest of researchers in expanding the knowledge on the therapeutic and pharmacological effects of these herbs (Jamal, 2006). A more scientific and modern approach, may earn these traditional medicinal plants a more respectable place in the community, as well as global acceptance.

The rainforest of Malaysia supports the growth of a rich diversity of valuable medical plants and herbs. *Ficus deltoidea*, locally known as Mas Cotek is one of the herbal plants that can be found in our rainforest. Traditionally, it is used in the form of leaves decoction for women after giving birth and is believed that it helps to contract the womb after deliver and regain strength. On top of that, the decoction is also used for the healing of various types of ailments such as fever and migraine (Ong *et al.*, 2011). Traditional practitioner claimed that the decoction is able to improve blood circulation and increase sexual behaviour (Caniago and Siebert, 1998). Like other traditional herb, Mas Cotek is playing an important role in herbal product business and is using by public for health maintenance (Musa, 2005). Not many research studies have been conducted on the plant previously. Only recent years, more studies have surface, researching on the effectiveness on the medicinal benefits of the plant.

1.2 Objectives

There are several studies showing the potential pharmacological effect of the aqueous extracts of *F. deltoidea*. However, most of the studies were focused on the phytochemical content of the plant extracts. There is no proteomic studies on the plant have been reported at the moment. Although plant proteomics have been advancing over the last decade, there are still challenges in herbal proteomics study due to the complexity of the sample and limitation of the genomic database. Protein profiling may provide an insight of the pharmacological pathway of the effect and the profiles can be used for authentication purposes. The aim of this study is to investigate and compare the proteins present in aqueous extracts of *Ficus deltoidea* var. *angustifolia, Ficus deltoidea* var. *trengganuuensis* and *Ficus deltoidea* var. *kunstleri* using proteomics techniques. This includes the following objectives.

1. To develop protein extraction method for the leaves aqueous extract of *F.deltoidea* for proteomic analysis.
2. To develop the proteome profiles of *F.deltoidea* extracts and compare using SDS-PAGE, 2-D gel electrophoresis and SELDI-MS.
3. To characterize the proteins separated in the proteome profiles of the three varieties of *F. deltoidea* leaves extracts.

Chapter 2 : Literature Review

2.1 Traditional Medicinal Plant

Medicinal plants are defined as plants that are commonly used in treating and preventing specific ailments and diseases, and that are generally considered to play a beneficial role in healthcare (Srivasta *et al.*, 1996). Since time immemorial, people have been using medicinal plants as their daily healthcare and in the amelioration of various ailments. In some places, it has been an alternative medicine to poor people as they are more affordable. They have been shown to have medicinal value with the effectiveness in prevention, alleviation and healing of various diseases and ailments. In addition, they are also well known for its gentle mean of managing chronic diseases, due to their less adverse effects compared to chemical drugs. 30 years ago, World Health Organzation (WHO) estimates that more than 80% of the population living in rural area rely either solely or largely in traditional remedies for healthcare (Bannerman, 1983). At present, 70 – 95 % of the population in developing country regularly use traditional medicine for the management of health and as primary health care. The use of herbal drugs is becoming more popular in the form of food supplement, nutraceuticals, and complementary and alternative medicine (Colegate *et al.*, 2007). It has been reported that 70 – 90 % of the population in some developed countries have used traditional medicines as an alternative to modern medicine (Robinson and Zhang, 2011). Various valuable medicinal plants that can be found in the rainforest of Malaysia are Tongkat Ali (*Eurycoma longifolia*), Kacip Fatimah (*Labisia pumila*), Hempedu Bumi (*Andrographis paniculata*), Mengkudu (*Morinda citrifolia*), Emas Cotek (*Ficus deltoidea*) and many others (Chang *et al.*, 2004).

2.2 *Ficus deltoidea*

F. deltoidea is one of the herbal plants discovered from our rich rainforest and is gaining popularity due to its medicinal values and health benefits. It is a well known herb used to facilitate womb contraction after giving birth. It is also believed that this herb cures various types of ailments and is good for health maintenance.

2.2.1 Classification

Taxonomy of *F. deltoitea* are shown in Table 1.1. This plant belongs to the family of Moraceae (Mulberry family). In Latin, it is called *Ficus deltoidea* Jack, which is synonym as *Ficus diversifolia* Blume (Bailey and Bailey, 1976). The common names are Mistletoe fig and Mistletoe rubber plant. The common name Mistletoe fig comes from the habit of the plant growing on other trees as an epiphyte. According to Starr *et al.* (2003), the scientific name *deltoidea* refers to the shape of the leaf.

Table 1.1 Taxonomy of *F. deltoidea*

Scientific Classification	
Kingdom	Plantae
Subkingdom	Viridiplantae
Phylum	Streptophyta
Class	Spermatopsida
Subclass	Magnollidae
Suberorder	Rosidae
Order	Rosales
Family	Moraceae
Tribe	Ficeae
Genus	*Ficus*
Species	*deltoidea*

Adapted from : http://www.uniprot.org/taxonomy/182111

2.2.2 Morphological Description of the Plant and Leaves of *F. deltoidea*

Plants in the genus *Ficus*, are all woody, ranging from trees and shrubs to epiphytes (Neal, 1965). In the wild, this species usually grows as an epiphyte, spreading and sprawling on larger trees with zigzagging branches (Riffle, 1998). It is seen as large cascading epiphytic shrub. It begins its life as an epiphyte. However, the plant is also found as evergreen shrub or small tree. It may attain a height of 15 - 22 feet (5 – 7 m) tall and 3 - 10 feet (1 – 3 m) wide (Brickell and Zuk, 1997). In Malaysia, it is usually found as a small shrub that grow up to 3 -5 meters. The leaves are usually 4 - 8 cm long, with different vartiety of shapes ranged from lanceolate or spoon-like shape (as shown in Figure 1.1(a)) to obovate or spatulate (as shown in Figure 1.1(b)) (Riffle, 1998). Obovate or spatulate shapes are more common than the other two. The ends of the spatulate-shaped leaves are ussually provided with a shallow notch. The leaves are leathery, thick and succulent with dark green above and rust to red olive brown beneath. The distinguishing feature of this plant are those golden yellow spot on the surface of the leaves and black or red spots in between the leaf veins at the back of the leaf. The bark is greyish and smooth, while the trunk is slender, and usually leaning. The plant bears spherical to round shape fruits which grows in pairs or solitary, axillary and ripening from dull yellow to orange or red (Brickell and Zuk, 1997). The size of the fruit is about 1.5 cm across and there is a light red spot on it (as shown in Figure 1.2).

According to traditional medical practitioners, *F. deltoidea* are classified into male and female plants based on the morphology of the plants. Plants with spoon-like shape leaves as shown in Figure 1.1(a) are classified as male plants. The back of the leaves have a reddish spot between the leaf veins. Usually there are only one red spot, but there are also leaves found to have 2 or 3 reddish spots. Male plants produce small yellowish fruits which turn orange when ripen (Figure 1.2(a)). Plants with bigger and thicker leaves (mostly round and oval in shape as shown in Figure 1.1(b)) are classified as female plants. The leaf base has black spots between the leaf veins instead of reddish spot as in male plant leaves. Besides, the leaf veins on female plant leaves are more obvious comparing to male plant leaves. Female plants produce round and green fruit which will turn red when ripen (Figure 1.2(b)).

Among ethnopharmacology researchers, *F. deltoidea* are further classified into several varieties based on the morphological variations of leaves and figs. Kochummen (1978) has described seven varieties that were found in Malaysia. They are *F. deltoidea* var. *kunstleri*, *F. deltoidea* var. *bilobata*, *F. deltoidea* var. *angustifolia*, *F. deltoidea* var. *deltoidea*, *F. deltoidea* var. *trengganuensis*, *F. deltoidea* var. *motleyana* and *F. deltoidea* var. *intermedia*. Fatihah *et al.* (2012) has suggested a morphological phylogenetic framework for these seven varieties of *F. deltoidea* which resolved the relationship of these varieties in *F. deltoidea* and also provides a basis for future molecular, cytological, phytochemical and pharmaceutical investigation. Another approach using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR - RFLP) to determine the variation or relationship in eight *F. deltoidea* samples has also been performed by Mahmod *et al*. (2011). However, the study shows that this method was not efficient as the detection method for genetic variations between varieties in *F. deltoidea*.

Figure 1.1(a) Front and rear view of male plant leaf

Male plant leaf is thin and small. Front view of the leaf is shown on the left, whereas the rear view is shown on the right. They usually have one red spot at the back of the leaf. The white strip indicated 1 cm of the actual scale.

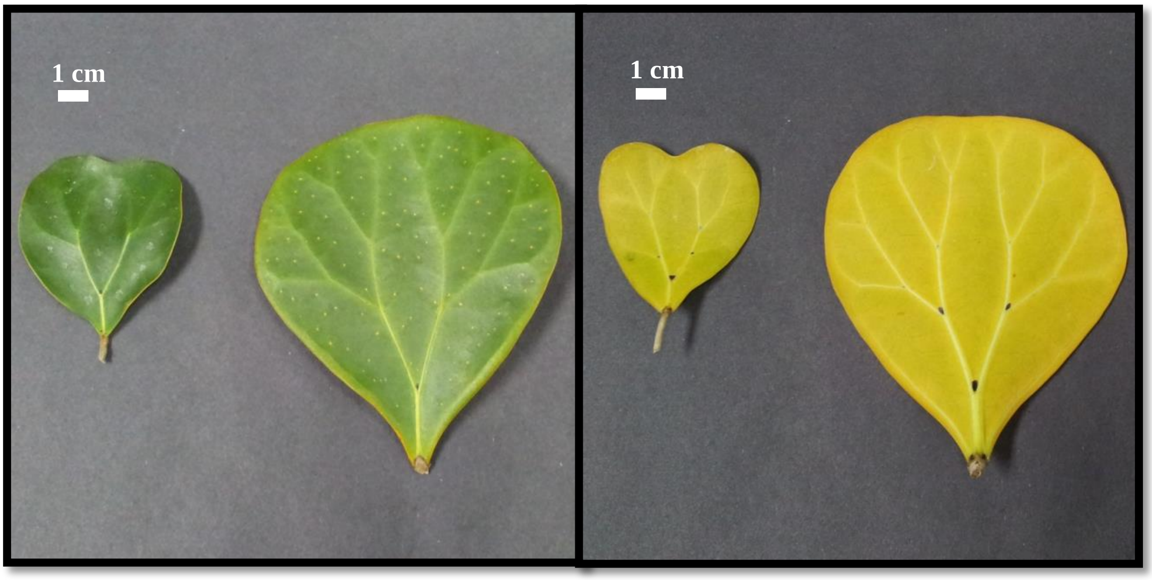


Figure 1.1(a) Front and rear view of female plant leaf

Female plant leaf is round and big. Their veins in the leaf are more obvious than male plant leaf. Front view of the leaf is shown on the left, whereas the rear view is shown on the right. The back of female plant leaf has three black spots between the leaf veins. The white strip indicated 1 cm of the actual scale.

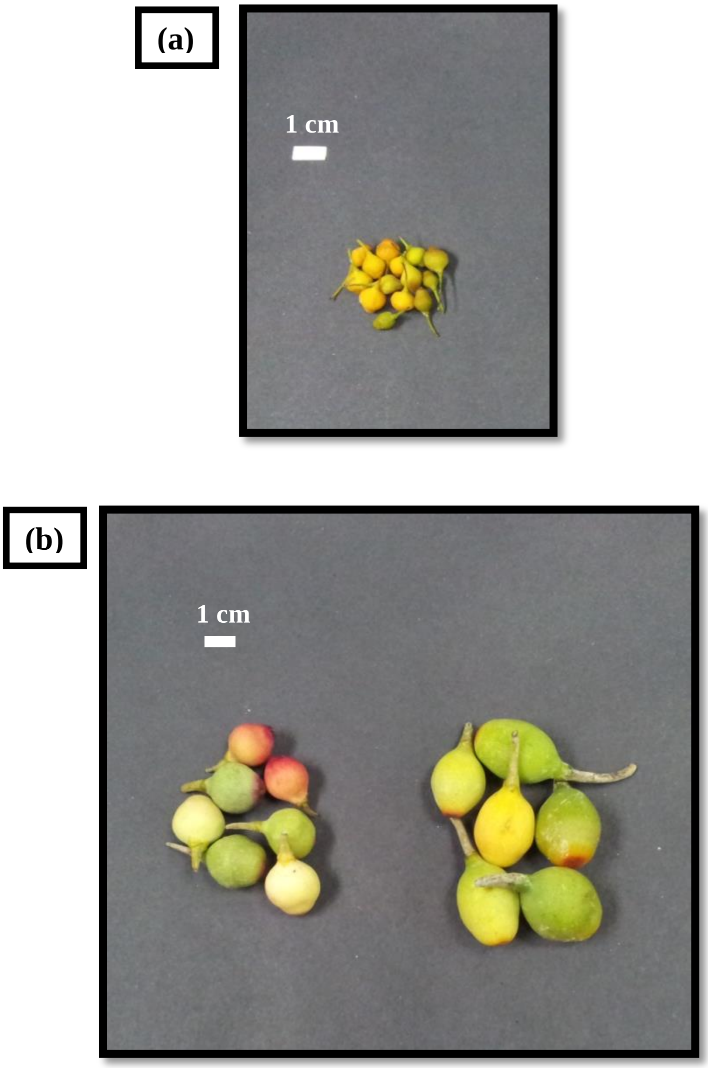


Figure 1.2 ***F. deltoideas* fruits produced from male and female plant**

F. deltoidea plant bears round to spherical fruits. Fruits produced from male plants are small and yellowish which will turn into orange when ripen as shown in (a). Female plant produces bigger green fruits which turn red when ripen (b).

2.2.3 Distribution of *F. deltoidea* Distribution

F. deltoidea is native to South East Asia which includes Borneo, Philippines, Indonesia and Malaysia, particularly Sabah and Sarawak. In Peninsular Malaysia, this plant is mostly found in eastern peninsular, especially in Kelantan and Terengganu. Other distribution in Malaysia also includes Perak (Relau Tujor (Wray) and Lumut), Pahang (Tahan River) Malacca (Ayer Keroh and Mt. Ophir), Selangor (Klang Gates) and Johor (Batu Pahat) (Ridley, 1967). It grows well in the forest near the beach and can thrive in the hill areas up to 700 meters from the sea level. Besides growing in wild, the species has been cultivated in various part of the world as a houseplant or an ornamental shrub (Starr *et al.*, 2003).

F. deltoidea is commonly known as Mas Cotek in Malaysia. The name 'Mas Cotek' is given by the local due to its feature of fine golden spots on the surface of each leaves. However, there are also other local names such as Delima Sudip, Serapat Angin, Telinga Beruk, Telinga Gajah and others. In the Borneo Island (Sabah, Sarawak and Kalimantan), this plant is known as 'Sempit-sempit' (Soepadmo and Saw, 2000). There are also ethnic groups in Sabah who called it 'Agoluran'. As for the Indonesian (Kalimantan), this plant is called 'Tabat Barito' and a number of names in other areas such as Ara Jelatih, Ara Tunggal, Api-Api Telinga Gajah and Api-Api Telinga Kera (Supriadi, 2000).

2.2.4 Traditional Beliefs

F. deltoidea has been traditionally used as an herb in the past by the locals of South East Asia. According to the '*Doctrine of signature*' concept, the shape, colour, taste, and smell of every part of plant *F. deltoidea* visualizes the uses of the herb in treating the diseases. Traditional medical practitioners used dried parts of the plant, including leaves, shoots and strips, boiled in water to obtained decoction of the plant. Decoction of the plant has been used as a remedy to treat various ailments and believed to have ability to promote good health (Musa, 2005). Besides that, the leaves and fruits can be chewed fresh. Continuous consumption of the fruits as 'ulam' is believed to maintain youth and vitality. Decoction of the leaves is consumed by women as a tonic and also to strengthen the uterus after giving birth (Anderson *et al*., 2003). It is believed that the herbal drink helps to return the womb to normal size. Decoction of the leaves is also claimed to be able to treat fever, migraine, improve blood circulation, and increase sexual behavior and fertility (Caniago and Siebert, 1998). The stem and barks of the plant are used as a remedy to cure disease such as pneumonia, diabetes mellitus, high blood pressure, skin disease and gout (Ong *et al.*, 2011). According to traditional medical practitioners, *F. deltoidea* is able to prevent and cure various ailments if practiced correctly. However, the effectiveness of this plant was not fully recognized yet due to lacks of scientific evidence to support these claims.

2.2.5 Phytochemical Contents

Studies on phytochemical contents of *F. deltoidea* showed that this herb contains flavonoids, tannins, triterpenoids, phenol and proanthocyanins (Abdullah *et al.*, 2009; Hakiman *et al.*, 2012). Phytochemicals that present in different part of the plant are shown in Table 1.2. Tannins, polyphenolic compound, triterpenoids and saponins are common compound in the leaves. Alkaloid is not present in the leaves but in the fruits and stem, except small-type leaves which have low amount of alkaloids content.

Flavonoid is a strong antioxidant and play important roles in maintaining healthy blood circulation, anti-inflammatory, anti-microbial and liver-protection (Pathak *et al.*, 1991; Draman *et al.*, 2012). It also gives yellow pigmentation to the plant. Tannins are any phenolic compound of sufficiently high MW containing sufficient hydroxyls and other suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied (Horvath, 1981). They are astringent and known with its functions in firming loose tissues and protect further tissue damage from edema and burns which contributes to anti-diarrhea, hemostatic and anti-hemorrhoidal activities (Vattem *et al.*, 2005). Condensed plant tannins could be also a treatment option for gastrointestinal inflammation caused by ulcerative colitis (Clinton, 2009). Phenols possess antiseptic and anti-inflammation characteristics, while triterpenoids can remove phlegm and aid nutrient adsorption. These compounds that are present may contribute to the medicinal properties of this plant.

Table 1.2 Chemical contents of *F. deltoidea*

Parts / Variety of *Ficus deltoidea*	**Chemical Constituents**
Leaves with big-type variety	Tannins, polyphenolic compound, triterpenoids, flavonoids, saponins.
Leaves with medium-type variety	Tannins, polyphenolic compound, triterpenoids, flavonoids, saponins.
Leaves with small-type variety	Tannins, polyphenolic compound, triterpenoids, alkaloids, saponins.
Fruits	Triterpenoids, alkaloids, flavonoids.
Stem	Alkaloids, flavonoids, saponins.

Obtained from: http://www.delto.com.my/

2.2.6 Research Studies

Pharmacology studies on *F. deltoidea* suggested potential hypoglycaemic effect of *F. deltoidea* leaves and fruits aqueous extracts. Research study conducted by Aminudin *et al.* (2007) demonstrated significant lowering of external glucose load in non-diabetic rats. Similar hypoglycaemic effect was also observed in mild diabetic rats (Adam *et al.*, 2007). The extracts of *F. deltoidea* are able to enhance both basal and insulin-stimulated glucose uptake into liver cells (Adam *et al.*, 2009). Also, aqueous extracts of *F. deltoidea* had demonstrated significant antinociceptive (Sulaiman *et al*., 2008) and anti-ulcerogenic (Zahra *et al.*, 2009) properties in laboratory animals. It is suggested that the extract is able to promote ulcer protection. Abdulla *et al*. (2009) showed the extracts of *F. deltoidea* possess promising antioxidant activity. Most of the studies showed that antioxidant activity of the extracts are associated with the high amount of polyphenols, phenolic acids and flavonoids (Aris *et al.*, 2009; Lee *et al.*, 2011; Omar *et al.*, 2011; Hakiman *et al.*, 2012). However, different varieties of *F. deltoidea* may contain different types of phenolic compounds and gave different antioxidant activities (Norra, 2011). Another study by Oh *et al.* (2011) showed the extract has strong anti-melanogenic activity and potentially to be used as a novel depigmenting agent for cosmetic. These studies suggested the presence of biologically active constituents that support the traditional use of this plant.

2.3 Proteomics

Proteomics is defined as the systematic analysis of the proteome, the protein complement of genome (Pandey and Mann, 2000). It is a large-scale study of proteins, particularly in their structures and functions, by biochemical methods. The set of proteins expressed in a specific cell, at a particular set of conditions is known as proteome. Proteins are the gene products that have undergone transcription, translation and post-translational modification. They are active agents involved in the metabolic and regulatory pathways in cells. Unlike genome which is a fixed characteristic of an organism, proteome changes with the stage of development, the environmental conditions of the organelle or tissue and the metabolic state. Proteomics research is a recent field that has been emerging over the past ten years because researchers are realizing that having complete sequences of genomes is not sufficient to elucidate the biological function. Proteomic gives a better assessment of the metabolic state of a cell and provides insight on how proteins affect cell processes.

At the beginning, proteomics is focused on the generation of protein profile and identification of the proteins (Wilkins *et al*., 1996). Today, the applications have been extended to the analysis of various functional aspects of proteins such as post-translational modification, protein-protein interactions, activities and structures. Proteomics has made a key contribution to the development of functional genomics. In medical research, introduction of proteomics have a significant impact on the development of future generations of diagnostic and therapeutic products. Detailed analysis of the proteome permits the discovery of new protein markers for diagnostic purposes and novel molecular targets for drug discovery (He and Chiu, 2003). Although proteomics research was initially focused on animals and yeast, nowadays these methods have been applied on plant materials.

This field of research is significantly influenced by the recent development in technologies. Protein separation and identification and characterization of resolved proteins are two important aspects in proteomics research. Separation of proteins from complex samples can be achieved by several techniques. However, the preferred methods are sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2-DE) and liquid chromatography (LC) (Šamaj and Thelen, 2007). Protein identification was typically done by the peptide mass fingerprinting using a MALDI-TOF instrument. Although there are many different methods emerging, the general technique of isolating and identifying the proteins remains largely unchanged.

2.3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Most of the electrophoretic methods used in protein separation nowadays are based on zonal or discontinuous electrophoresis in polyacrylamide gels (Simpson, 2003). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a variation of discontinuous electrophoresis. Electrophoresis is a technique which separates charged molecules based on their mobility in an applied electrical field. When charged molecules are placed in an electric field, they will migrate towards either positive or negative electrode according to their charges. The mobility of the molecules is influenced by their total charge, size and shape. Polyacrylamide gels act as the molecular sieves for molecules in electrophoresis. This porous matrix is formed from the polymerization of acrylamide monomer and small amount of bis-acrylamide. By varying the concentration of acrylamide and the cross-linker, different pore size of polyacrylamide gel can be formed. In the presence of SDS, proteins dissociate and unfold into polypeptide chains coated with negatively charged SDS molecules. SDS molecule binds to polypeptides at a constant weight ratio (Reynolds and Tanford, 1970), giving them identical charge densities. Therefore the electrophoretic migration rate of the proteins through a gel is determined only by the size, which is the molecular weight. This enable the fractionation of protein mixtures by molecular weight as larger proteins move through the gel slower compared to smaller proteins due to sieving effect of the gel. SDS-PAGE has been used in prognostic purpose (Bazzi *et al.*, 1997), authentication (Ashurst and Dennis, 1998; Aiken *et al*., 1998), qualitative and quantitative analysis of complex mixtures of proteins (Sadeghi *et al*., 2006). Preparative SDS-PAGE was also used as a rapid purification method of enzymes (Mohammadian *et al*., 2010). Also, molecular weight of the proteins can be estimated by running marker proteins of known molecular weight simultaneously (Weber and Osbom, 1969).

At present, there are several SDS-PAGE buffer systems which are commonly used. SDS-PAGE buffer system described by Laemmli (1970) is the most cited electrophoresis method in life sciences (Chiou and Wu, 1999). Different range of proteins can be separated by adjusting the acrylamide concentration. However, Laemmli buffer system has low resolving power for proteins below 10 kDa. Tricine-SDS-PAGE introduced by Schägger (1987) has advantages for separating small proteins and peptides below 30 kDa (Schägger, 2006). Combining two electrophoresis buffer systems enable the separation of proteins ranged from 1 – 500 kDa (Schägger, 2006). Nevertheless, there is limitation on the resolving power of SDS-PAGE for complex protein mixtures. Overlapping of protein bands might occur in highly complex protein mixture. Phinney and Thelen (2004) study revealed the possibility of a single band in the SDS-PAGE of a complex protein sample may consists of several proteins with similar molecular weight. Development of two-dimensional gel electrophoresis had overcome the shortcomings of SDS-PAGE.

2.3.2 Two-Dimensional Gel Electrophoresis

The concept of two-dimensional gel electrophoresis (2-DE) was pioneered by O'Farrell (1975). This technique separates proteins in two steps, based on two independent properties. It begins with the first dimension separation of proteins based on their pI using isoelectric focusing (IEF), followed by second dimension fractionation by SDS-PAGE based on their molecular weight. IEF is an electrophoretic technique where proteins are separated in a pH gradient. The pH at which a protein has no net electric charge is known as pI. The original methodology described by O'Farrell suffered from some drawbacks due to the difficulties in obtaining reproducible pH gradient tube gels. Introduction of immobilized pH gradient (IPG) strips together with their commercialization has greatly improved the reproducibility and resolution for this separation method (Görg *et al.*, 2000), which resulted in major resurgence in 2-DE.

Since 2-DE separation is solely based on the physiochemical properties of the proteins, it can present a steady and high resolution protein expression pattern in the biological samples under specific metabolic state. This powerful resolving capability of 2-DE has made it one of the core technologies for the analysis of complex mixtures extracted from biological samples. More than 1000 different proteins were able to be resolved in a 2-DE protein map (O'Farrell, 1975). Highly reproducible reference map from 2-DE enable quantitative analysis of the protein expression pattern of cell or tissue samples at different pathological state or under different experimental conditions, such as various drugs or chemical treatments. For example, 2-DE was used to resolve the protein expression under different abiotic stress in order to study stress regulation process of grasspea (Chattopadhyay *et al*., 2011).

Despite being a powerful separation technique, it suffers from some limitations as well. Hydrophobic membrane proteins are often under represented on 2-DE gels due to the inability to solubilize highly hydrophobic proteins (Rabilloud, 1998). Basic proteins are poorly resolved by 2-DE due to several reasons (Görg *et al*., 2000). Visualization of low abundance proteins on 2-DE gels is also one of the challenges in 2-DE. A typical plant cell may contains more than 20,000 polypeptides, while a maximum of only 3000 spots can be resolved and observed in a typical 2-DE gel (Lilley *et al*, 2001). This results in the domination of abundant proteins in 2-DE map. Hence, optimization becomes an important factor in getting high resolution protein map. Several efforts have been made to enhance the resolution of 2-DE (Görg *et al.*, 2004). The dynamic range of proteins can be improved through fractionating proteome into specific sub-proteome (Bae *et al.*, 2003). The used of gradient polyacrylamide gels are able to enhance the sharpness of protein bands (Margolis and Kenrick, 1969).

2.3.3 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique used to measure the molecular weight of a sample or molecule based on mass-to-charge (*m/z*) ratio. Basically, a mass spectrometer consists of 3 parts; ion source, mass analyser and detector. The basic principle of MS involves ionization of the sample molecules, fragmentation of the charged molecules and finally measurement of their mass-to-charge ratios. Mass spectrometry analysis are carried out in gas phase, thus sample analytes need to be vaporized. Before 1980s, MS analysis was restricted to small molecular weight and thermally stable compounds due to the lack of effective techniques to ionize high molecular weight compounds and biological samples. In 1990s, biological mass spectrometry emerged as a powerful analytical method in protein analysis (Pandey and Mann, 2000). The emergence of mass spectrometry-based proteomics was driven by the development of two ionization methods, electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) (Aebersold and Mann, 2003). These techniques enabled the ionization of proteins without significant fragmentation, thus made proteins accessible to mass spectrometry analysis. ESI generates ionized analytes from liquid samples and therefore is usually coupled with liquid-based separation tools, such as high performance liquid chromatography (HPLC). Conversely, MALDI sublimates and ionizes analytes from a solid phase. According to Domon and Aebersold (2006), ESI is less tolerant of contaminant compared to that of MALDI. In present day, application of MS in proteomics includes characterization and quality control on macromolecules and recombinant proteins, protein identification and also characterization and detection of post-translational modification that alter the mass of a protein (Mann *et al*., 2001).

2.3.4 Matrix-Assisted Laser Desorption/Ionization

Matrix-Assisted Laser Desorption/Ionization (MALDI) is first introduced by Karas and Hillenkamp (1988). Its ability of nondestructive vaporization and ionization of biomolecules from solid material has made it a valuable analytical tool for protein analysis. MALDI is usually coupled to time-of-flight (TOF) analyser for molecular mass determination (Aebersold and Mann, 2003). In MALDI analysis, protease digested protein samples are first co-crystallized with a matrix solution on a metal target. The matrix is a UV-absorbing weak organic acid such as α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) or dihydroxy benzoic acid (DHB). When a UV laser is fired at the matrix-peptide mixture, the matrix strongly absorbs the laser light energy and eventually leads to desorption of the matrix together with the peptides into gas phase. The matrix plays a key role in assisting the vaporization and ionization of peptides. It facilitates ionization of the peptides by acting as a proton donor and receptor (Lewis *et al*., 2000). The energy is passed from the matrix to the peptide in a gentle manner so that ions of intact peptides are produced without fragmentation. Ionized peptides are then subjected to TOF analyser, where the ions are separated according to their *m/z* ratio. The *m/z* of ions is calculated from the time taken by the ions to travel down TOF tube. Small ions are lighter, thus they have higher velocity and reach the detector in a shorter time compared to larger ions. The detector records and translates signals from the ions into a mass spectrum.

Protein identification is achieved by a process called peptide-mass fingerprinting. This idea was first proposed in 1977 by Cleveland *et al.*, on the protein identification of viral-coat protein, using the in-gel proteolysis masses of peptides estimated from SDS-PAGE. In 1993, the combination of peptide mapping and MS was introduced (James, 2000). The concept is to digest a protein with enzymes or chemicals that specifically cleave at certain amino acids to produce a set of peptides. Exact masses of the peptides were determined by MALDI-TOF, forming a mass fingerprint for that particular protein. Protein identity is assigned by matching the experimental mass fingerprint against a list of peptide masses generated by theoretical fragmentation of a protein in a database. Target protein is usually purified by two-dimensional gel electrophoresis prior to enzyme digestion. High accuracy and sensitivity of MALDI-TOF instrument has made it core technology in proteomics. Applications of MALDI-TOF in proteomics include post-separation identification and as a robust tool of disease biomarker.

2.3.5 Surface-Enhanced Laser Desorption/Ionization

Surface-Enhanced Laser Desorption/Ionization (SELDI), introduced by Hutchens and Yip (1993), is a variation of MALDI. The ability of SELDI-TOF to analyse complex protein mixtures based on their binding affinity make it unique from other MS-based system. The protein chip arrays are the heart of the SELDI-TOF technology. The protein chips possess chromatographic surfaces that are uniquely designed to retain proteins from complex mixtures based on their specific properties. Protein chip arrays can be composed of chemically or biologically active surfaces. Protein chips with hydrophobic, cationic, anionic, metal ions or hydrophilic surface are known as "chemical surface" and are mostly used for protein expression profiling studies. Another series of protein chips with biological active surfaces incorporate immobilized antibody, receptor, DNA and enzyme, can be use in protein interaction studies (Seibert *et al.*, 2004). The principle of ionization in SELDI is similar to MALDI. Captured proteins co-crystallized with matrix on the surface of protein chips is ionized with a nitrogen laser and their molecular masses are measured by TOF.

One of the popular applications of SELDI-TOF is in the discovery of potential biomarkers by identifying the different protein expression pattern in clinical samples (Jr *et al.*, 1999). It has shown great potential as a promising diagnosis tool in early detection of diseases, such as breast cancer (Hu *et al*., 2005). The increase popularity in diagnostic proteomics is due to its advantages in allowing rapid analysis of proteins in small sample volumes and enable the profiling of low MW proteins (less than 20 kDa) (Issaq *et al.*, 2003). In addition, SELDI-MS has higher sensitivity compared to 2-DE-MS approach and is a potential alternative for 2-DE to overcome the limitations on the isolation of low abundant and hydrophobic proteins (Seibert *et al*., 2004). Characterizing protein-protein and protein-DNA interaction studies can also be done using SELDI-MS technology (Stoica *et al.*, 2001; Hinshelwood *et al.*, 1999).

2.4 Plant Proteomics

While proteomics research is advance in animal and yeast, progress in plants has been more recent. Before the development of MS, most of the studies were more on determination of possible protein markers for different genotype and phenotype using 2-DE. The availability of nucleotide sequence information and the development of mass spectrometry in protein identification have open up new perspective for analysing plant proteome and their complex functions. Many plant biology laboratories used proteomics methods to comparatively profile plant protein expression and identify plant proteins. The research objectives have ranged from the proteomic analysis of organ, tissue, cell and subcellular fractions to the study of plant developmental processes, the responses to various stresses and post-translational modification of the proteins and protein interactions (Jorrín *et al*., 2009). Over the past few years, there are increases in the research conducted on specific subcellular proteome, especially in chloroplast, mitochondria and membranes. Plant proteomics were also applied to monitor different developmental changes and the responses of environmental stimuli on protein expressions. Comparative proteomics were also conducted on proteome of mutant and the complement in wild-type. Identification of those proteins not only provides new insights into protein localization, but also information on plant biological processes such as responses to stress, mechanism of transportation and metabolism, secretory pathway and seed germination. These studies have been reviewed in Park (2004), Rossignol *et al.* (2006) and Jorrín *et al.* (2009). Although 2-DE-MS approach remains to be the dominant analytical method, "second-generation" proteomic techniques such as difference gel electrophoresis (DIGE), liquid chromatography-mass spectrometry (LC-MS), multidimensional protein identification technology, isotope-coded affinity tags and stable isotope labeling by amino acids in cell culture have started to make an impact (Rossignol *et al.*, 2006).

Most of the plant proteomics researches were on *Arabidopsis thaliana* (L.) Heynh. and rice (*Oryza sativa* L.), which have a more advance genome project. The first plant proteomic work was published on *Arabidopsis*, where the proteomes of leaf, stem, root, seed and callus were profiled. Among the 4763 protein separated, 136 proteins were sequenced (Kamo *et al.*, 1995). Subsequently, more studies on *Arabidopsis* via proteomics approach have emerged. For instance, protein localization, vacuole function, signal transduction pathways and biogenesis of *Arabidopsis thaliana* were studied through detailed analysis and characterization of the proteome (Slabas *et al.*, 2004; Carter *et. al*., 2004; Hwang *et al.*, 2002). In the study carried out by Bae *et al.* (2003), nuclear proteome of *Arabidopsis* were profiled using 2-DE and resulted in 500-700 protein spots resolved on 2-DE gel. Furthermore, changes of the nuclear proteome in response to cold stress were analysed. Identification and characterization of the up- and down-regulated proteins provide an insight of a variety of cellular function in the cell. Similar study on stress response by proteomics approach was also carried out on rice (Yan *et. al*., 2005; Zou *et al*., 2011). Proteomics studies on rice had provide an insight of molecular mechanism in response to stress and also revealed the development process of grain filling and seed maturation (Komatsu and Tanaka, 2005). Since crop productivity was greatly depends on the ability to adapt, resist and tolerate variable environmental condition, these studies might have contribute to the improvement of the crops and detection of novel traits for breeding.

2.5 Challenges in Plant Proteomics

Generally, proteomics studies on plant are more challenging than cell or serum because other than plant proteins, plant cell contains proteases and compounds that would interfere with protein extraction and proteomic analysis (Tsugita and Kamo, 1999). These include polysaccharides, starch, lipids and various secondary metabolites. The presence of proteases during plant protein extraction may cause protein degradation and thus resulted in artifactual spots in 2-DE map. Vascular plant contains large amount of secondary metabolites accumulated in the vacuole, especially phenolic compounds. Phenolic compounds can bind irreversibly with proteins, oxidized by phenoloxidases and lead to streaking in 2-D gels (Wang *et al.*, 2008). Similarly, polysaccharides and lipids can also cause severe disturbance in proteomics analysis (Görg *et al.*, 2004). The rigidity of plant cell walls also contributes to the challenges of plant proteomics. Plant proteome is highly complex and dynamic. Highly dynamic range of plant proteins make proteomics analysis of all protein components becomes difficult. Certain tissues may contain highly abundant proteins, for instant ribulose bisphosphate carboxylase /oxygenase in leaves, which may dominate protein profile. This hindered the proteomic assessment of low molecular weight proteins in 2-DE gel (Xi *et al.*, 2006). Hydrophobic membrane proteins in plant cell post a major challenge in their proteomics analysis. Most of the hydrophobic proteins are not soluble in non-detergent isoelectric focusing sample buffer and tend to precipitate during isoelectric focusing, which lead to significant losses of the proteins (Ephritikhine *et al.*, 2004).

Various strategies have been developed to overcome these challenges. Fractionating a proteome into sub-proteome according their biochemical, biophysical and cellular properties was able to reduce the overall complexity, enhance the coverage and the detection of certain group of proteins such as membrane proteins and low-abundant proteins (Park, 2004). It also has advantage to overcome the limitation of proteomic techniques. Sample preparation is a critical step in proteomics studies to obtain optimal results. Protein precipitation is one of the strategies to enhance proteomic analysis by removing contaminants and concentrate the proteins. Several protein extraction protocols such as TCA/acetone precipitation, phenol-based extraction, chloroform/methanol fractionation, ammonium sulfate precipitation and polyethylene glycol (PEG) fractionation were available. TCA and acetone precipitation appeared to be one of the common and effective protocols used for plant samples (Askari *et al.*, 2006; Plomion *et al.*, 2006; Yan *et al.*, 2006; Xu *et al.*, 2008). It has the advantage in the inhibition of proteases, phenoloxidases and peroxidases (Wang *et al.*, 2008). It is also very useful in minimizing protein degradation and removing interfering compounds, such as polyphenols and salt (Görg *et al.*, 2004). However, there are some drawbacks such as difficulty in dissolving the resulting pellet, precipitation of nucleic acids longer than 20 nucleotides and hydrolyzation of some proteins by TCA (Chen and Harmon, 2006). Ammonium sulfate precipitation is known for its antichaotropic and mild nature towards proteins. It has an advantage over all other techniques in the stability of protein. High salt concentration prevents proteolysis and bacterial action. Hence, protein precipitated is usually stable for years (Scopes, 1994). Due to the diversity of protein abundance, molecular weight, charge, hydrophobicity, post-translational modifications and complication with other molecules, there is no single efficient protein extraction protocol that can capture the entire proteome (Wijk, 2001). Selecting suitable buffer to solubilize protein extract is important in obtaining high resolution and reproducible results. Improvements in the solubilization of hydrophobic proteins have been made with the use of thiourea (Rabilloud, 1998) and new zwitterionic detergents (Rabilloud *et al.*, 1990; Chevallet *et al.*, 1998; Babu *et al.*, 2003).

2.6 Proteomics Applications on Traditional Herbal Medicine

Although ancient herbal medicine prescriptions have been used for centuries to maintain health and treat various ailments, the target molecules and underlying mechanism of their effects remain largely unclear. Introduction of proteomics into traditional herbal medicine had allowed better understanding of such complex mixtures and the reputed medicinal effects at the cellular and molecular levels. To date, there are a number of researches on traditional medicinal plant using proteomics techniques, especially in traditional Chinese medicinal plant. Most of the medicinal plant studies based on proteomics techniques were focused on the pharmacological effects of the extracts and their molecular mechanism involved. A study on the hemopoiesis stimulation effect of Siwu decoction was investigated via proteomic techniques (Guo *et al.*, 2004). Differential proteome analysis of blood marrow in blood deficiency mice revealed that Siwu decoction could regulate some proteins and enzymes involved in the hemopoiesis system, which explain the underlying mechanism on promoting hemopoisis. The mechanisms behind the effects of *Ganoderma lucidum* spores in promoting neural regeneration were revealed through proteomics study on rats (Zhang *et al.*, 2006). Moreover, traditional medicinal plant authentication could be done through proteomic studies. For example, a proteome approach was used to identify proteins in *Panax ginseng* and *Panax quinquefolius* and biomarkers that enable to distinguish different parts of the ginseng (Lum *et al.*, 2002). It shows the potential of proteomics technologies as an identification tool for various medicinal plants that might be difficult to identify morphologically or anatomically. Authentication by proteomics studies has allowed quality control of herbal medicine (Zeng *et al.*, 2008) and probably further understanding of the physiological mechanisms.

Chapter 3 : Materials and Methods

3.1 Materials

3.1.1 Chemicals and Solvents

All chemicals used were purchased from Merck Sdn. Bhd., except for CHCA, bromophenol blue sodium salt for electrophoresis, 25 % glutaraldehyde, coomassie blue G-250, PVPP and Folin-Ciocalteu reagent were from Sigma-Aldrich. Trypsin used for MALDI analysis was Trypsin Gold, Mass Specreometry Grade (Promega, USA) purchased from DKSH Sdn. Bhd. IPG buffer and cover fluid oil for strip rehydration used were from GE Healthcare. Milli-Q water was obtained from Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya.

3.1.2 Apparatus and Consumables

Apparatus used in this project includes disposable 96-well plate, cloth coffee strainer, stainless steel tea strainer, beaker, centrifuge tubes, reswelling tray (GE Healthcare) and gradient maker SG 30 (Amersham Hoefer). Paper wick and 11 cm IPG strip (non-linear, pH 3 – 10) were obtained from GE Healthcare. ProteinChip® H50 and NP20 arrays were obtained from Biorad, USA. SnakeSkin Dialysis Tubing (3.5K MWCO) was purchased from Thermoscience, USA whereas ZipTip-C18 was purchased from Merck Millipore.

3.1.3 Specific Software

Three software used in this project were ProteinChip Data Manager Software 3.5 (Bio-rad), Melanie 7.0 (GeneBio) and Global Protein Server Explorer 3.6 Software (Applied Biosystem).

3.1.4 Kits

Two kits were used in this project. They were Pierce® BCA Protein Assay Kit (Thermoscientific, USA) and Pierce™ Glycoprotein Staining Kit (Thermoscientific, USA). Pierce® BCA Protein Assay Kit contains BCA Reagent A (1000 mL), BCA Reagent B (25 mL) and 2 mg/mL Albumn Standard Ampules (10 X 1 mL). On the other hand, Pierce™ Glycoprotein Staining Kit contains glycoprotein stain (250 mL), oxidizing reagent (2.5 g), reducing reagent, (1.25 g), horseradish peroxide (1 mg) and soybean trypsin inhibitor (1 mg)

3.1.5 List of Equipment

- Electronic balance (Mettler Toledo)
- Biofuge Stratos Highspeed Table Top Centrifuge (Heraeus)
- Ettan IPGphor II system (GE Healthcare), including
    - Manifold tray (GE Healthcare)
- Eyela MicroTube Pump MP-3 (Eyela)
- FreeZone 18 Litre Console Freeze Dry Systems (Labconco, US)
- Grinder
- Hot plate stirrer (Fisher Scientific)
- Image Scanner III (GE Healthcare)
- SE 600 electrophoresis system (Amersham Hoefer)
- Sunrise™ Absorbance Reader (*Tecan Austria GmbH)*
- Microprocessor pH Meter (Hanna Instrument)
- SDS-PAGE gel set (C.B.S. Scientific Co.), including,
    - Glass plates
    - Spacer

- Rubber
- Dual Vertical Mini-Gel Unit

- Electrophoresis Power Supply (Scie-Plas)
- Orbital Shaker (Protech)
- TS Series Ultra-Low Temperature Freezer (Thermo Scienctific)
- Water bath (Memmert)
- SpeedVac Concentrator (Thermo Scientific)
- ProteinChip® SELDI system, Enterprise Edition (Bio-rad, USA)
- MALDI-TOF/TOF mass spectrometer, ABI 4800 plus (Applied Biosystems)

3.1.6 Plant Materials

Three varieties of *Ficus deltoidea* were supplied by Delto Medicama Plantation (M) Sdn. Bhd. Leaves of the three varieties include the small-type leaves (SL) or *F. deltoidea* var. *angustifolia* (KLU 046467), medium-type leaves (ML) or *F. deltoidea* var. *trengganuuensis* (KLU 046469) and big-type leaves (BL) or *F. deltoidea* var. *kunstleri* (KLU 046470) (Figure 3.1). Voucher specimens of the leaves has been deposited in Herbarium, University of Malaya. Air dried leaves were ground into powder form using grinder and stored in dry place.

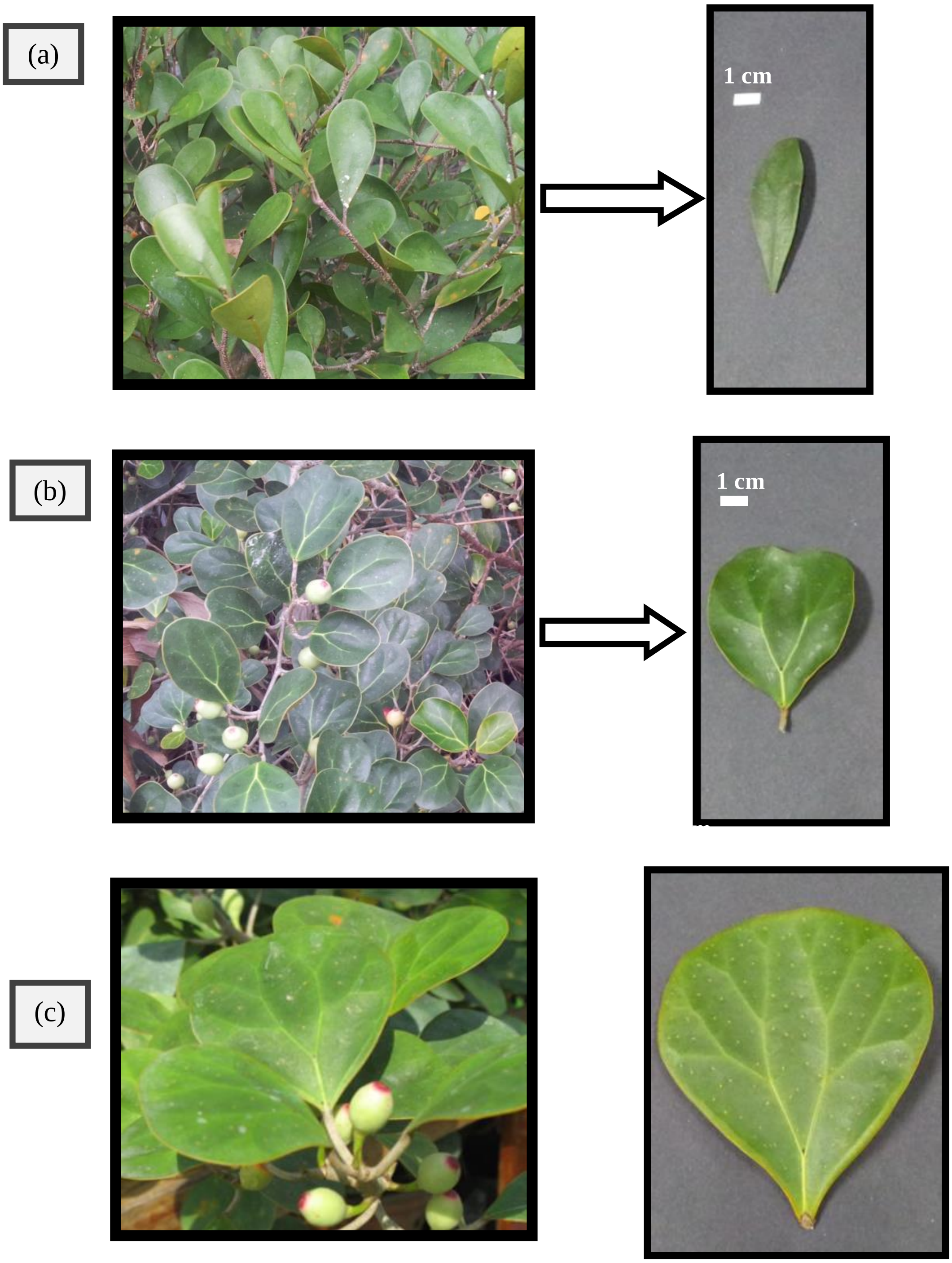


Figure 3.1 Different varieties of *F. deltoidea* plant

Varieties of *F. deltoidea* used in this experiment were (a) SL or *F. deltoidea* var. *angustifolia,* (b) ML or *F. deltoidea* var. *trengganuuensis* and (c) BL or *F. deltoidea* var. *kunstleri*. Specimens of the leaves has been deposited in Herbarium, University of Malaya.

3.2 Sample Preparation

3.2.1 *F. deltoidea* Decoction Preparation

Dried plant samples were decocted in aqueous in a ratio of 1 : 10. One hundred gram of powdered leaves was weighed and added into 1 L of hot distilled water. Samples were boiled for two hours. After two hours, another 1 L of hot distilled water was added into the mixture and boiled for another two hours (second extraction). After the extraction, crude extracts were filtered with tea strainer and cloth coffee strainer. Subsequently, aqueous extracts were concentrated to a lower volume at approximately 60 °C. Concentrated extracts were then centrifuged at 5000 rpm for 5 min followed by filtration using filter paper. Finally, aqueous extracts were subjected to freeze-drying by Labconco FreeZone 2.5 freeze-dryer at Faculty of Science, University of Malaya. Extracts were frozen in - 80 °C freezer prior to the freeze-drying process.

3.2.2 PVPP treatment

200 mg of freeze-dried crude extract powder (SL, ML and BL) were weighed separately and dissolved in 5 mL distilled water to make a crude extracts of 40 mg/mL concentration. 1 % (w/v) of polyvinylpyrrolidone (PVPP) was added into the crude extracts and incubated at 4 °C for approximately 4 hours. After 4 hours, the extracts were filtered by filter paper to remove the PVPP. Subsequently, the extracts were subjected to protein extraction method described in section 3.3.1 until 3.3.4. Finally, the yield of freeze-dried protein extracts obtained for each sample were calculated.

3.3 Protein Precipitation Procedure

3.3.1 Ammonium sulfate precipitation (Method 1)

Crude aqueous extracts were centrifuged at 5000 rpm for 10 min. The supernatant was proceeded with ammonium sulfate precipitation. Amount of ammonium sulfate required was calculated using EnCor Biotechnology Inc. calculator (http://www.encorbio.com/protocols/AM-SO4.htm). An amount of 3.11 g ammonium sulfate (90 % saturation) were weighed and slowly added into 5 ml aqueous extracts. The aqueous extracts were continuously stirred until all the added salt were dissolved. Then, the solutions were incubated for 4 hours at 4 °C. The solutions were centrifuged at 10,000 x g at 4 °C for 15 min to pellet out protein precipitate. Precipitate collected after centrifugation were re-dissolved in distilled water to yield protein solutions. Protein solutions were transferred into dialysis tube with 3,500 Da molecular cut off point (Thermoscience, USA) and dialysed against distilled water for 48 hours. Extracts were subjected to freeze-drying after dialysis.

3.3.2 Cold acetone precipitation (Method 2)

First of all, acetone was pre-cooled at – 20 °C. Crude extracts were mixed with cold acetone in a ratio of 1 : 4. Five millilitres of extracts were mixed with 20 mL of cold acetone and incubated at – 20 °C for overnight. Samples were centrifuged at 12,000 x g for 15 min at 4 °C. Pellets were collected and re-dissolved in distilled water. Protein samples were aliquot in microcentrifuge tubes and freeze-dried.

3.3.3 TCA/acetone cold precipitation (Method 3)

Crude aqueous extracts were subjected to TCA/acetone precipitation method as described in Sheoran *et al.* (2009). TCA/acetone solution containing 10 % (w/v) TCA and 0.07 % (v/v) β-mercaptoethanol was prepared by dissolving 10 g of TCA in 100 mL acetone containing 0.07 % (v/v) β-mercaptoethanol. One volume of sample was mixed with 4 volume of TCA/acetone solution to precipitate the proteins. Twenty millilitres of TCA/acetone solution were added to 5 mL of extracts and incubated at – 20 °C for overnight. Samples were centrifuged at 12,000 x g for 15 min at 4 °C to pellet down the protein precipitate. The pellets were washed with 80 % acetone containing 0.07 % β-mercaptoethanol. Then, protein precipitates were re-dissolved in distilled water and aliquot in microcentrifuge tubes. Finally samples were freeze-dried.

3.3.4 Ammonium sulfate precipitation combined with acetone/β-mercaptoethanol precipitation (Method 4)

Aqueous extracts were subjected to ammonium sulfate precipitation as the method described in section 3.3.1. However, dialyzed extracts were proceeded with acetone washing prior to freeze-drying. Extracts were mixed with acetone containing 0.07 % β-mercaptoethanol in a ratio of 1 : 4. Twenty millilitres of cold acetone containing 0.07 % β-mercaptoethanol were added to 5 mL of aqueous extracts and mixed thoroughly. The mixtures were incubated at – 20 °C for overnight. Samples were centrifuged at 12,000 x g for 15 min at 4 °C to pellet down precipitated proteins. Pellets were re-dissolved in distilled water and aliquoted in microcentrifuge tubes, then subjected to freeze-drying.

3.4 Protein Content Measurement

3.4.1 Preparation of chemical solutions

(i) Sample extract

1 mg/mL sample solution was prepared by dissolving 20 mg protein extract in 20 mL distilled water.

(ii) Preparation of diluted Albumin (BSA) Standards

Different final BSA concentration were prepared according to the guidance in Table 3.1. The concentration of stock BSA solution was 2.0 mg/mL.

(iii) Preparation of Working Reagent

Working Reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part or BCA Reagent B.

3.4.2 Protein quantitation by Pierce® BCA protein assay

Protein assay was performed according to the protocol provided in Pierce® BCA Protein Assay Kit instruction manual. 25 µL of blank, standard and sample solution were pipetted into the wells in microplate. 200 µL of Working Reagent were added into each well and mixed well. The plate was incubated at 37 °C for 30 min. After incubation, the microplate was cooled to room temperature and the absorbance value were measured at 562 nm on Sunrise™ Absorbance Reader (Tecan Austria GmbH). BSA standard curve was plotted and protein concentrations of the protein extracts were determined from the equation $y = 0.001x$ obtained from the standard curve. Protein content in 1 mg protein extract was calculated. Percentage of protein content in crude protein was determined by dividing protein content (in µg) with 1000 µg protein extract and multiply with 100 %.

Table 3.1 Preparation of BSA standards

Final BSA Concentration	Tube	Volume of distilled water	Volume of BSA
2,000 µg/mL	A	125 µL	300 µL of Stock
1,500 µg/mL	B	325 µL	375 µL of Stock
1,000 µg/mL	C	175 µL	325 µL of Stock
750 µg/mL	D	325 µL	175 µL of tube B dilution
500 µg/mL	E	325 µL	325 µL of tube C dilution
250 µg/mL	F	325 µL	325 µL of tube E dilution
125 µg/mL	G	400 µL	325 µL of tube F dilution
25 µg/mL	H	400 µL	100 µL of tube G dilution
0 µg/mL (Blank)	I	400 µL	-

3.5 Total Phenolic Content Measurement

3.5.1 Preparation of chemical solutions

(i) 7.5 % (w/v) Sodium carbonate solution

75 g of sodium carbonate was dissolved in 1 L distilled water.

(ii) 200 mM Folin-Ciocalteu reagent

Stock Folin-Ciocalteu reagent was diluted 10 times with distilled water.

(iii) Various concentration of gallic acid solution

1 mg/mL stock solution of gallic acid was prepared by dissolving 5 mg of gallic acid powder in 5 mL distilled water. Different concentrations of gallic acid ranged from 1 to 125 µg/ml were prepared as described in Table 3.2.

Table 3.2 Preparation of different concentration of gallic acid solutions.

Concentration (µg/ml)	Gallic acid stock solution (mL)	Distilled water (mL)
1	0.005	4.995
5	0.025	4.975
10	0.050	4.950
25	0.125	4.875
50	0.250	4.750
75	0.375	4.625
100	0.500	4.500
125	0.625	4.375

3.5.2 Total phenolic content determination

Total phenolic content in the samples were determined using the protocol described in Song *et al* (2010). Firstly, 500 µL of samples and gallic acid solutions were mixed with 2.5 mL 200 mM Folin-Ciocalteu reagent in test tubes and incubated for 4 minutes. Subsequently, 2 mL of 7.5 % (w/v) sodium carbonate was added into each tube. The absorbance readings were measured at 760 nm after 2 hours of incubation at room temperature. Average absorbance values for each gallic acid solution were plotted against their respective absorbance value. Total phenolic content of samples were determined from the equation $y = 0.0105x + 0.0126$ and presented in mg/g of plant material.

3.6 SDS-PAGE Analysis

3.6.1 Preparation of buffer solutions for Laemmli SDS-PAGE system

(a) <u>Solution A : 30 % T, 0.8 % C Monomer stock solution</u>

60 g of acrylamide and 1.20 g of N,N'-methylenebisacrylamide were dissolved in 80 mL distilled water and was top up to 250 mL in a measuring cylinder. This solution was kept in the dark at 4 °C.

(b) <u>Solution B : 4 X Resolving gel buffer solution [1.5 M Tris base, pH 8.8]</u>

36.35 g of tris base was dissolved in 150 mL distilled water and adjusted to pH 8.8 with concentrated HCl. The solution was then top up to 200 mL.

(c) <u>Solution C : 10 % (w/v) SDS</u>

5 g of SDS was dissolved in 50 mL distilled water.

(d) Solution D : 10 % (w/v) APS

100 mg of APS was weighed and dissolved in 1 mL distilled water. This solution was freshly prepared before use.

(e) Solution E : TEMED

This solution was used directly from the commercial bottle.

(f) Solution F : 4 X Stacking buffer solution [0.5 M Tris base, pH 6.8]

6.06 g of tris base was dissolved in 80 mL distilled water and adjusted to pH 6.8 with concentrated HCl. The solution was then top up to 100 mL.

(g) 1 % (w/v) Bromophenol blue

100 mg of bromophenol blue and 60 mg of tris base were dissolved in 10 mL of distilled water.

(h) 2 X Sample buffer [125 mM Tris, 4 % (w/v) SDS, 20 % Glycerol, 10 % (v/v) β-mercaptoethanol, 0.05 % (w/v) Bromophenol blue, pH 6.8]

2 mL of Solution F, 3.2 mL of Solution C, 1.6 mL of glycerol, 0.8 mL of β-mercaptoethanol and 0.4 mL of 1 % (w/v) bromophenol blue were mixed well.

(i) Laemmli SDS electrophoresis running buffer [25 mM Tris, 0.192 M Glycine, 0.1 % (w/v) SDS]

3.30 g of tris base, 14.86 g of glycine and 1.0 g of SDS were dissolved in 800 mL distilled water and top up to 1000 mL.

3.6.2 Preparation of buffer solutions for Tricine-SDS-PAGE system

(a) Solution A : 30 % T, 0.8 % C Monomer stock solution

Solution A was prepared as described in section 3.6.1(a).

(b) Solution D : 10 % (w/v) APS

Solution D was prepared as described in section 3.6.1(d).

(c) Solution E : TEMED

This solution was used directly from the commercial bottle.

(d) Solution G : 3 X Gel buffer solution [3 M Tris base, 0.3 % (w/v) SDS, pH 8.45]

36.34 g of tris base and 0.30 g of SDS were dissolved in 80 mL distilled water and adjusted to pH 8.45 with concentrated HCl. This solution was then top up to 100 mL with distilled water and stored at 4 °C.

(e) 0.5 M Tris-HCl, pH 8.45

6.06 g of tris base was dissolved in 80 mL distilled water and adjusted to pH 8.45 with concentrated HCl. Finally, it was then top up to 100 mL with distilled water.

(f) 2 X Tricine sample buffer [100 mM Tris-HCl, 4 % (w/v) SDS, 24 % (w/v) Glycerol, 0.02 % (w/v) Coomassie Blue G-250, 4 % (w/v) beta-mercaptoethanol, pH 8.45]

400 mg of SDS, 3.455 g of 87 % glycerol and 2 mg of coomassie blue G-250 were dissolved and mixed well in 2 mL of 0.5 M Tris-HCl, pH 8.45. Finally, 400 µL of β-mercaptoethanol was added and made up to 10 mL with distilled water.

(g) Anode buffer [0.2 M Tris-base, pH 8.9]

24.228 g of tris base was first dissolved in 800 mL distilled water. The solution was adjusted to pH 8.9 with concentrated HCl and top up to 1 L with distilled water.

(h) Cathode buffer [0.1 M Tris-base, 0.1 M Tricine, 0.1 % (w/v) SDS]

12.114 g of tris base, 17.92 g of tricine and 1 g of SDS were dissolved and made up to 1 L with distilled water.

3.6.3 Preparation of stacking and separating gel

Stacking and separating gel mixtures were prepared according to Table 3.3 and 3.4. Solution D and E were added prior to use. Separating gel mixture was transferred into the gel plate sandwich using pipette and subsequently 500 µL distilled water was layered on top of the gel. It was then left on the bench to polymerize. When the gel was polymerized, the water layer was removed and replaced with stacking gel mixture. A gel comb was inserted into stacking gel layer and again left to polymerize on the bench. Gel surface of polymerized stacking gel was then rinsed with distilled water and running buffer before proceeds with gel electrophoresis.

3.6.4 Sample preparation and SDS-PAGE analysis

Samples were mixed with 2 X sample buffer at a ratio of 1 : 1 and incubated for 4 minutes at 90 °C. 10 µL of samples and 5 µL of peptide standard were loaded into wells at stacking gel layer. Electrophoresis run was started at 60 V and increased to 100 V when the dye front entered the separating gel. The electrophoresis was stopped once the dye front reached at about 1 cm from the bottom of the gel. The gels were removed from the gel set and subsequently visualized by silver staining.

Table 3.3 Composition of stacking and separating gel for Laemmli SDS-PAGE

Solutions		Stacking gel 4 % T, 3 % C	Separating gel 18 % T, 3 % C
Solution A	(mL)	0.65	12.00
Solution B	(mL)	-	5.00
Solution C	(µL)	50.00	200.00
Solution D	(µL)	25.00	100.00
Solution E	(µL)	5.00	6.60
Solution F	(mL)	1.25	-
Distilled water	(mL)	3.05	2.70
Total volume	**(mL)**	**5.00**	**20.00**

Table 3.4 Composition of stacking and separating gel for Tricine-SDS-PAGE

Solutions		Stacking gel 4 % T, 3 % C	Separating gel 16 % T, 3 % C
Solution A	(mL)	0.485	6.79
Solution D	(µL)	48.00	105.00
Solution E	(µL)	6.00	7.00
Solution G	(mL)	2.00	7.00
Distilled water	(mL)	3.515	4.638
Total volume	**(mL)**	**6.00**	**21.00**

3.7 Post-Electrophoresis Gel Visualization Using Silver Staining

3.7.1 Preparation of chemical solutions

(a) 5 % (w/v) Sodium thiosulfate

0.5 mg of sodium thiosulfate was dissolved in 10 mL of distilled water.

(b) Fixing solution [40 % (v/v) Ethanol, 10 % (v/v) Acetic Acid]

40 mL of ethanol was mixed with 10 mL of acetic acid and made up to 100 mL with distilled water.

(c) Sensitizing solution

First of all, 0.2 g of sodium thiosulfate and 6.8 g of sodium acetate were dissolved in distilled water. 30 mL of ethanol and 500 µL of 25 % glutaraldehyde were then added into the solution and made up to 100 mL with distilled water.

(d) Silver solution

0.25 g of silver nitrate was dissolved in distilled water. 40 µL of 37 % formaldehyde were added prior to use and made up to 100 mL with distilled water.

(e) Developing solution

2.5 g sodium carbonate was weighed and dissolved in distilled water. 40 µL of 37 % formaldehyde and 2.8 µL of 5 % sodium thiosulfate were added prior to use and made up to 100 mL.

(f) Stopping solution

1.46 g of EDTA-disodium salt dihydrate was dissolved and made up to 100 mL with distilled water.

3.7.2 Silver staining procedure

Immediately after electrophoresis, gels were fixed in fixing solution for 30 minutes. The fixing solution was then replaced with sensitizing solution and incubated for another 30 minutes. Subsequently, the gels were washed three times with large amount of distilled water for 5 minutes. After the washing step, gels were incubated in silver solution for 20 minutes. Next, gels were washed twice with distilled water approximately for 1 minute to remove unbound silver. Developing solution was added and incubated between 5 to 10 minutes. Once spots or bands were visualized, developing solution was immediately replaced with stopping solution and incubated for 10 minutes. Those gels were then washed three times with distilled water for 10 minutes and finally scanned using Image Scanner III (GE Healthcare).

3.8 Glycoprotein Detection

Glycoprotein staining was performed using Pierce^{TM} Glycoprotein Staining Kit (Thermoscientific, USA) to detect the presence of glycoproteins. *F. deltoidea* samples, horseradish peroxide (positive control) and soybean trypsin inhibitor (negative control) were first subjected to SDS-PAGE and the gel obtained was proceeded with glycoprotein staining.

3.8.1 Preparation of chemical solutions

(a) <u>3 % Acetic acid</u>

3 % Acetic acid was prepared by mixing 30 mL of acetic acid with 970 mL of ultrapure water. This solution was stored in room temperature.

(b) 50 % Methanol

1 L of 50 % methanol was prepared by adding 500 mL of methanol into 500 mL of ultrapure water.

(c) Oxidizing Solution

250 mL of 3 % acetic acid was added to dissolve material in the “Oxidizing Reagent” bottle, provided in the Glycoprotein Staining Kit.

(d) Reducing Solution

250 mL of ultrapure water was added to dissolve material in the “Reducing Reagent” bottle, provided in the Glycoprotein Staining Kit.

3.8.2 Glycoprotein staining procedure

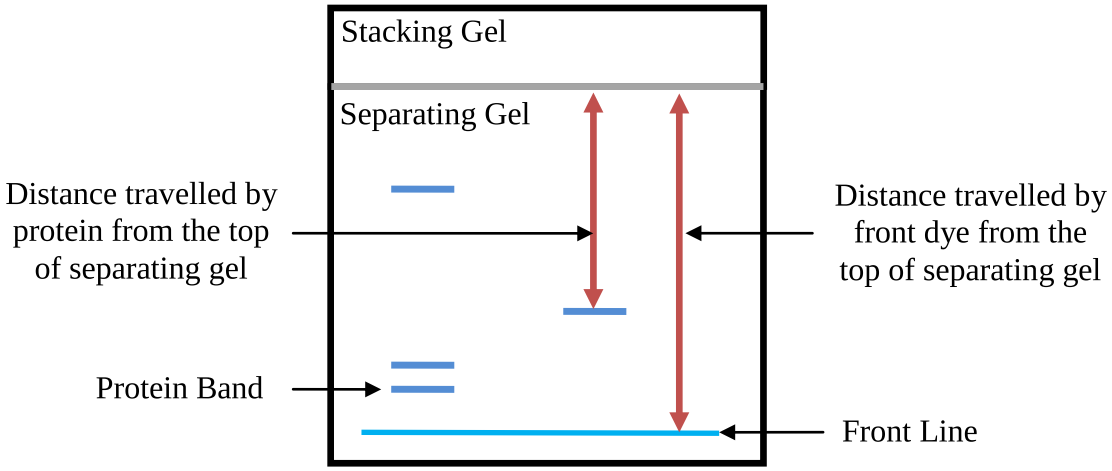
SDS-PAGE gels were fixed in 100 mL of 50 % methanol for 30 minutes after electrophoresis. Subsequently, gels were washed twice in 100 mL 3 % acetic acid with gentle agitation for 10 minutes. Next, gels were incubated in 25 mL Oxidizing Solution for 15 minutes, followed by three times washing with 100 mL of 3 % acetic acid for 5 minutes. After the washing, gels were stained with 25 mL Glycoprotein Staining Reagent for 15 minutes. Glycoprotein Staining Reagent was replaced with 25 mL Reducing Solution and incubated for 5 minutes with gentle agitation. Finally, gels were washed extensively with 3 % acetic acid and then with distilled water. Stained gels were scanned and compared with silver stained SDS-PAGE profile.

3.9 Protein MW Estimation on SDS-PAGE profile

Molecular weight (MW) of the protein bands in SDS-PAGE gels were determined by comparing their motilities to the motilities of known protein markers. R_f values of known MW standard makers and each proteins were calculated using the equation stated below.

$$R_f = \frac{\text{distance travelled by protein from the top of separating gel}}{\text{distance travelled by front dye from the top of separating gel}}$$

A calibration curve was generated by plotting the logarithm of protein molecular masses against the R_f values. MW of protein bands in each sample was determined using the equation obtained from calibration curve.



3.10 Two-Dimensional Gel Electrophoresis Separation

Two-dimensional gel electrophoresis separation consists of two step, namely first dimensional isoelectric focusing (IEF) and second dimensional SDS-PAGE. First dimensional isoelectric focusing was performed using Ettan IPGphor II system (GE Healthcare), according to protocol recommended by the manufacturer with modification. Protein samples were seperated by isoelectrophoresis using 11 cm IPG strip (GE Healthcare) with non-linear pH 3 - 10. Second dimensional separation was performed on 10 - 18 % gradient SDS-PAGE gel by SE 600 electrophoresis system (Amersham Hoefer).

3.10.1 Preparation of chemical solutions

(a) Urea rehydration stock solution [7 M Urea, 2 M Thiourea, 2 % (w/v) CHAPS, 0.5 % (v/v) IPG buffer, 0.002 % (w/v) Bromophenol blue]

10.50 g of urea, 3.80 g of thiourea and 0.50 g of CHAPS were dissolved in 20 mL distilled water. 125 µL of IPG buffer and 50 µL of 1 % (w/v) bromophenol blue were added and top up to 25 mL with distilled water.

(b) Rehydration sample buffer [7 M Urea, 2 M Thiourea, 4 % (w/v) CHAPS, 40mM Tris, 65 mM DTT, 0.002 % (w/v) Bromophenol blue]

4.20 g of urea, 1.52 g of thiourea, 0.40 g of CHAPS, 48.44 mg tris base and 100 mg of DTT were dissolved in 5 mL distilled water. 10 µL of 1 % (w/v) bromophenol blue was added and top up to 10 mL with distilled water.

(c) SDS Equilibration buffer [6M Urea, 2 % (w/v) SDS, 70 mM Tris-HCl, pH 8.8, 0.002 % Bromophenol blue]

72.1 g of urea, 4 g of SDS and 84.2 g of glycerol were dissolved in 100 mL of distilled water. 10 mL of 4 X resolving buffer and 400 µL of 1 % (w/v) bromophenol blue were added and top up to 200 mL. This buffer was kept at – 20 °C for storage purpose. Before proceed to the preparation of SDS equilibration buffer I and II, the buffer was thawed at room temperature until all the crystalized urea were dissolved.

(d) SDS equilibration buffer I

100 mg of DTT was dissolved in 10 mL of SDS equilibration buffer

(e) SDS equilibration buffer II

250 mg of iodoacetamide was dissolved in 10 mL of SDS equilibration buffer.

(f) Agarose sealing solution [25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS, 0.5 % (w/v) agarose, 0.002 % (w/v) bromophenol blue]

0.5 g of agarose was dissolved in 100 ml of Laemmli SDS electrophoresis buffer. 200 µl of 1 % (w/v) bromphenol blue was added into the solution and boiled until the solution become clear and homogenize.

(g) Cathode buffer (Laemmli SDS electrophoresis buffer)

Cathode buffer was prepared as described in section 3.7.1 (i).

(h) Anode buffer [375 mM Tris-HCl, pH 8.8]

First, 181.6 g of tris base was dissolved in 3 L of distilled water. The pH of the solution was adjusted to 8.8 with concetrated HCl. Finally, the solution was top up to 4 L with distilled water.

3.10.2 Preparation of 10 - 18 % gradient gel

Glass plates were assembled according to the user's manual provided by the manufacturer. For gradient gel, two different percentage of gel mixture were prepared; 18 % acrylamide gel solution (heavy solution) and 10 % acrylamide gel solution (light solution). Both heavy and light solutions were prepared according to Table 3.5. 10 - 18 % gradient gel was casted using gradient maker SG 30 (Amersham Hoefer). The gradient maker was connected to a peristaltic pump (Eyela MicroTube Pump MP-3) through a tubing with a tip at the other end. Heavy and light solutions were filled in separate chambers of the gradient maker. Once the valve of the gradient maker was opened, gel mixture of heavy and light solution was pumped through the tubing into the glass plate sandwich. Finally, the gel mixture was overlaid with distilled water and allowed to polymerize.

3.10.3 Rehydration of IPG strip and IEF

IPG strips were first rehydrated with urea rehydration buffer. 200 µL of urea rehydration solution was slowly pipetted into the slots in reswelling tray to prevent bubble formation. Using a forcep, drystrips were carefully placed on top of the sample mixture with the gel surface facing down. Subsequently, the slot was overlaid with drystrip coverfluid. The strips were incubated passively for 16 - 18 hours at room temperature. On the next day, rehydrated strips were transferred from the reswelling tray to Ettan IPGphor II (GE Healthcare). Strips were placed in the Manifold tray for Ettan IPGphor II with the gel surface facing up folowing the correct orientation. Paper wicks were pre-wetted and placed overlapping the gel at both ends of the IPG strips. Electrodes were positioned at both ends of the strips and sample cups were placed near the anode. Then, coverfluid oil was added into slots that contains IPG strip. 250 µg of protein samples was mixed with 125 µL of rehydration sample buffer and centrifuged at 5,000 rpm for 10 minutes to remove undissolved materials. 100 µL of clear protein sample mixtures was loaded in the sample cup and layered with cover fluid oil. First dimension IEF was performed using the voltage setting listed in Table 3.6, at 20 °C, 50 µA per strip. After the run was completed, strips were rinsed with Milli-Q water and stored at – 80 °C or proceeded to the second dimension gel electrophoresis.

3.10.4 Equilibration of IPG strips

Prior to second dimension gel electrophoresis, IPG strips were equilibrated with equilibration buffer. Equilibration was carried out in two steps; reduction (step 1) and alkylation (step 2). Strips were first equilibrated in equilibration buffer with DTT for 15 minutes, followed by another 15 minutes re-equilibration in equilibration buffer with iodoacetamide with continuous shaking. In each step, 5 ml of equilibration buffer was used per strip. Following this step, equilibration buffer was discarded and the strips were rinsed with cathode buffer. Subsequently, strips were placed on top of gradient gel surface and sealed with pre-melted 0.5 % agarose solution.

3.10.5 Second dimension gel electrophoresis procedure

Once the agarose solution has polymerized, gel electrophoresis set was assembled and filled with cathode and anode buffer. The gel apparatus was connected to a chiller which maintained the gel separation system at a constant temperature of 16 °C. Second dimension gel electrophoresis was performed at 40 µA and 25 W per gel, 50 V for 30 minutes and 600 V until dye front reached 1 cm above the bottom of the gel. Electrophoresis was stopped and glass plates were disassembled. A cut was made at the positive corner of the gel in order to indicate the orientation of the gel. The gel plugs were removed and subsequently proceed with silver staining as described in section 3.7. The silver staining procedure was the same except for a few changes listed below.

1. Distilled water was replaced with Milli-Q water.
2. 25 % glutaraldehyde in sensitizing solution was excluded.
3. 37 % formaldehyde in silver solution was excluded.

Milli-Q water has high purity and is deionised. Ionic and bacteria contamination can significantly affect the results in MALDI MS/MS analysis. Therefore, it is important to use ultrapure deionised water to obtain clean spectra. Glutaraldehyde and formaldehyde encourage protein reticulation by methylene bridges which lead to peptide loss during protein digestion (Richert *et al.*, 2004).

Subsequently, stained gels were analysed using Melanie 7.0 (GeneBio) to detect the presence of protein spots. Selected spots were further analysed by mass spectrometry.

Table 3.5 Composition of light and heavy solutions for gradient gel

Solutions	Light Solution (10%)	Heavy Solution (18%)
Acrylamide (mL)	13.333	24.00
4 X Resolving Buffer (mL)	10.00	10.00
Distilled water (mL)	16.067	Top up to 40ml
10% SDS (µL)	400.00	400.00
10% APS (µL)	200.00	200.00
TEMED (µL)	13.20	13.20
Sucrose (g)	-	6.00
Total (mL)	40.00	40.00

Table 3.6 Voltage setting for first dimension IEF by Ettan IPGphor II

Steps	Condition	Voltage (V)	Total voltage hours (Vhrs)
1	Step and hold	100	100
2	Step and hold	500	500
3	Step and hold	1000	1000
4	Step and hold	8000	12500
Total voltage hours			14100

The experiment was conducted at 20 °C with 50 µA per strip

3.11 MALDI-TOF-TOF Analysis

3.11.1 Preparation of chemical solutions

(a) 200 mM Ammonium bicarbonate buffer stock solution

1.5811 g of ammonium bicarbonate was dissolved in 100 mL Milli-Q water.

(b) 100 mM Ammonium bicarbonate buffer solution

200 mM ammonium bicarbonate solution was diluted to 100 mM by adding 1 volume of Milli-Q water into 1 volume of stock buffer solution.

(c) Destaining solution [15 mM Potassium ferricyanide in 50 mM sodium thiosulfate]

74.05 mg sodium thiosulfate and 49.389 mg potassium ferricyanide were dissolved in 10 mL Milli-Q water.

(d) Reducing buffer [100 mM DTT in 100 mM ammonium bicarbonate]

15.43 mg DTT was dissolved in 10 mL of 100 mM ammonium bicarbonate.

(e) Alkylating buffer [55 mM Iodoacetamide in 100 mM ammonium bicarbonate]

101.73 mg of iodoacetamide was measured in a Falcon tube. 10 mL of 100 mM ammonium bicarbonate was added into the Falcon tube to dissolve the iodoacetamide.

(f) Washing buffer [100 mM Ammonium bicarbonate in 50 % acetonitrile]

20 mL of acetonitrile was mixed with 20 mL of 200 mM ammonium bicarbonate solution.

(g) 50 mM Acetic acid

28.6 µL of acetic acid was mixed with 9971.4 µL Milli-Q water.

(h) Elution Buffer [50 % Acetonitrile / 0.1 % TFA]

500 µL acetonitrile and 10 µL TFA were mix into 9.49 mL of Milli-Q water.

(i) Equilibration buffer [0.1 % TFA]

10 µL TFA was added into 9.99 mL of Milli-Q water.

(j) Trypsin buffer [10 % Acetonitrile in 50 mM ammonium bicarbonate]

250 µL of 200 mM ammonium bicarbonate and 100 µL of acetonitrile were mixed well with 650 µL of Milli-Q water.

(k) Trypsin solution [7 ng/µL]

100 µg of trypsin was reconstituted in 100 µL of 50 mM acetic acid to obtain 1 µg/µL trypsin stock solution. Stock solution of trypsin was aliquoted into 1.5 µL microcentrifuge tube, with each tube containing 5 µL of the trypsin solution and stored at – 20 °C. Prior to use, aliquoted trypsin solution was thawed on ice and diluted to 7 ng/µL by adding 695 µL of trypsin buffer into the tube.

(l) Matrix buffer [60 % Acetonitrile / 0.1 % TFA]

600 µL acetonitrile and 10 µL TFA were mix into 9.39 mL of Milli-Q water.

(m) Matrix solution (CHCA)

10 mg of CHCA was dissolved in 1 mL of matrix buffer and vortexed for 2 minutes. The matrix solution was spun at 1,000 x g for 1 minute. Supernatant was transferred into a new microcentrifuge tube and kept in the dark.

3.11.2 In-gel digestion

Protein spots were cut and placed in 1.5 mL centrifuge tubes. Gel plugs were first destained with 50 µL of destaining solution with constant shaking. After 30 minutes, solution in the tube was aspirated and replaced with 100 µL reducing buffer. Gel plugs in reducing buffer were incubated at 60 °C for another 30 minutes. Subsequently, reducing buffer was replaced with 100 µL of alkylating buffer and incubated in the dark for 20 minutes. Gel plugs were then washed with 500 µL of washing buffer for 20 minutes with constant shaking. Washing steps were repeated twice. After washing, 50 µL of acetonitrile was added into the tubes and shook for 15 minutes to shrink the gels. Gel plugs were then dried using SpeedVac Concentrator (Thermo Scientific) for 15 minutes. 25 µL trypsin solution was added into each tube to reswell the gel plugs and incubated overnight at 37 °C to digest the proteins. On the next day, tubes were spun briefly and solutions in the tube were tranferred into new tubes (Tube A). 50 µL of elution buffer were added and vortexed for 15 minutes. Supernatants were then again transferred into Tube A. 50 µL of acetonitrile were added into the tubes and vortexed for 15 minutes. Finally, supernatants were pooled together in Tube A and dried using SpeedVac Concentrator (Thermo Scientific). Dried peptides were then reconstituted in 10 µL of equilibration buffer before proceeded to ziptip cleanup procedure.

3.11.3 ZipTip cleanup procedure, sample spotting and MALDI analysis

ZipTip-C18 (Millipore) was used for sample cleanup. ZipTip-C18 pipette tip was attached to a 10 µL micropipette and the volume setting of the micropipette was adjusted to 10 µL. First, ZipTip column was washed with acetonitrile by carefully aspirating the acetonitrile through the ZipTip column. The solution was dispensed slowly until it reached the upper surface of the column. It is important not to let the tip to run dry. This step was repeated twice. Next, the column was equilibrated 5 times with equilibration buffer. Slowly, the tip was transferred into tubes containing digested peptides, followed by repeated aspiration and dispensed of the sample solution for approximately 10 cycles to bind the sample to the column. Subsequently, the column was rinsed 5 times with equilibration buffer to wash out salts and other water-soluble impurities. Finally, digested sample bound to the column was eluted in 1.5 µL elution buffer. All the steps were done carefully and slowly to prevent introducing air bubbles into the ZipTip. Before spotting onto MALDI plate, 1.5 µL of matrix solution was added and mixed well. 0.7 µL of the sample-matrix solution was spotted on the MALDI plate and allowed to dry. Once dried, the last step was repeated and sample was applied onto the same spot. MALDI analysis was performed by MALDI-TOF/TOF mass spectrometer, ABI 4800 plus (Applied Biosystems). Peptide mass tolerance and fragment mass tolerance were set at 100 ppm and 0.2 Da respectively. Data obtained from spectra were analysed by Global Server Explorer 3.6 software (Applied Biosystems) and searched against *Ficus* species database in NCBI database.

3.12 SELDI-TOF Analysis

3.12.1 Preparation of buffers and matrix

(a) <u>Binding buffer [0.5 % (v/v) TFA in 50 % (v/v) Acetonitrile]</u>

5 mL of acetonitrile and 50 µL of TFA were added into 4.95 mL Milli-Q water.

(b) <u>Washing buffer [50 % (v/v) Methanol]</u>

50 % methanol was prepared by adding 1 volume of 100 % methanol with 1 volume of Milli-Q water.

(c) <u>Preparation of Matrix solution (CHCA)</u>

7 mg of CHCA was dissolved in 200 µL of binding buffer and vortexed for 2 minutes. The matrix solution was spun at 1,000 x g for 1 minute. Supernatant was transferred into a new microcentrifuge tube and kept in the dark.

3.12.2 Sample preparation and SELDI analysis procedure

ProteinChip® H50 (Bio-rad, USA) was used in this experiment. ProteinChip® arrays were inserted into array cassette and placed in the bioprocessor following the correct orientation. Spots on the array were washed twice with washing buffer for 5 minutes. 10 µL of acetonitrile was spotted onto the spots until dryness, followed by pre-wetting with 5 µL of binding buffer. Subsequently, pre-wetting solution was replaced with 2.5 µL of sample. ProteinChips® were incubated at room temperature until the sample were semi-dried. 2 µL of matrix solution was added on each sample spot and allowed to dry completely. Arrays were analsed using ProteinChip® SELDI system, Enterprise Edition (Bio-rad, USA) using the protocol setting stated in Table 3.7. Once spectra were generated, they were subjected to baseline substraction, peak detection and normalization using ProteinChip Data Manager Software 3.5 (Bio-rad). Peaks were detected with the condition of > 5.0 S/N for both valley depth and peak height. Peak intensities were normalized to total ion current intensity in 2 – 15 kDa to compensate spectrum-to-spectrum variations. Peak clustering was carried out with the setting of first-pass peak detection S/N > 5.0 (for both valley depth and peak height), second-pass peak detection of S/N > 2.0, 10 % minimum peak threshold and a mass window approximately 0.1 % of peak mass. Then, peaks were subjected to Expression Difference Mapping (EDM) analysis to calculate the *P* value across different precipitation methods and different plant variety.

Table 3.7 Protocol setting for SELDI-TOF analysis

Acquisition setting	
Mass Range	0 – 20,000 Da
Focus mass	5,000 Da
Matrix Attenuation	500 Da
Sampling Rate	800 MHz
Laser Energy	1200 nJ
Warming Shot	1320 nJ
Shots kept	795

Chapter 4 : Results

4.1 Hot Water Extraction

Decoctions of the leaves of SL, ML and BL were dried by freeze-drying process. After dehydration process, dry crude extracts were obtained in the form of brown powder as shown in Figure 4.1. ML extract has a slightly darker brown colour compared to SL and BL extracts. Dry weight of the crude extracts obtained were measured and compared. Decoction of 100 g SL, ML and BL leaves yielded 16.83 ± 0.85 g, 12.13 ± 0.68 g and 12.50 ± 1.15 g of crude extracts respectively. Comparison of the crude extract yielded from three varieties of *Ficus* leaves were shown in Figure 4.2. Decoction of SL leaves gave slightly higher crude extract yield compared to ML and BL. Whereas ML and BL leaves extraction gave almost same amount of crude extract.

4.2 PVPP Treatment

Reconstitution of the crude extracts gave a dark brown solution as shown in Figure 4.3(A). Before proceed with protein precipitation, crude extracts were treated with PVPP to remove tannins and other polyphenolic compounds. Changes of the extract's colour were observed after incubation with PVPP. Figure 4.3(B) showed the extracts become lighter in colour following the PVPP treatment. Total phenolic content in the extracts of pre- and post-PVPP treatment was also being measured and presented in Figure 4.5. Gallic acid was used as a reference standard and the standard curve was shown in Figure 4.4. Total phenolic content in pre-PVPP treatment extracts of SL, ML and BL were measured at 77.69 ± 0.26 mg/g, 85.69 ± 0.47 mg/g and 80.61 ± 0.78 mg/g plant material. After PVPP treatment, total phenolic content in SL, ML and BL extracts were reduced to 52.64 ± 0.68 mg/g, 61.97 ± 0.37 mg/g and 49.34 ± 0.91 mg/g respectively. PVPP treated samples were subsequently subjected to different precipitation methods.

Figure 4.1 Freeze dried crude extract of *F. deltoidea* leaves

Brown powder extracts were obtained after freeze-drying. In the picture, SL, ML and BL represent *F. deltoidea* variety with small-type leaves, medium-type leaves and big-type leaves respectively. The colour of ML extract was slightly darker than the other two.

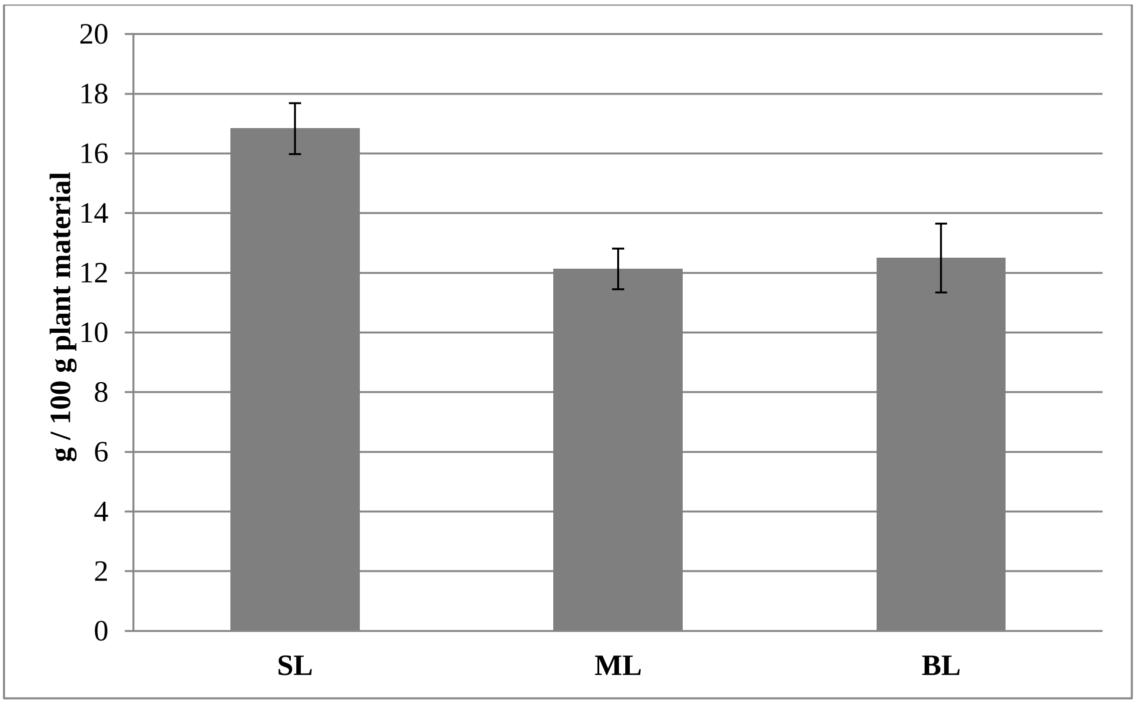


Figure 4.2 Comparison of crude extracts yielded from hot water extraction of *F. deltoidea* leaves

SL leaves has the highest crude extract yield, which is 16.83 ± 0.85 g/100 g plant material. Crude extracts yielded of ML and BL leaves were 12.13 ± 0.68 g/100 g and 12.50 ± 1.15 g/100 g plant material, respectively.

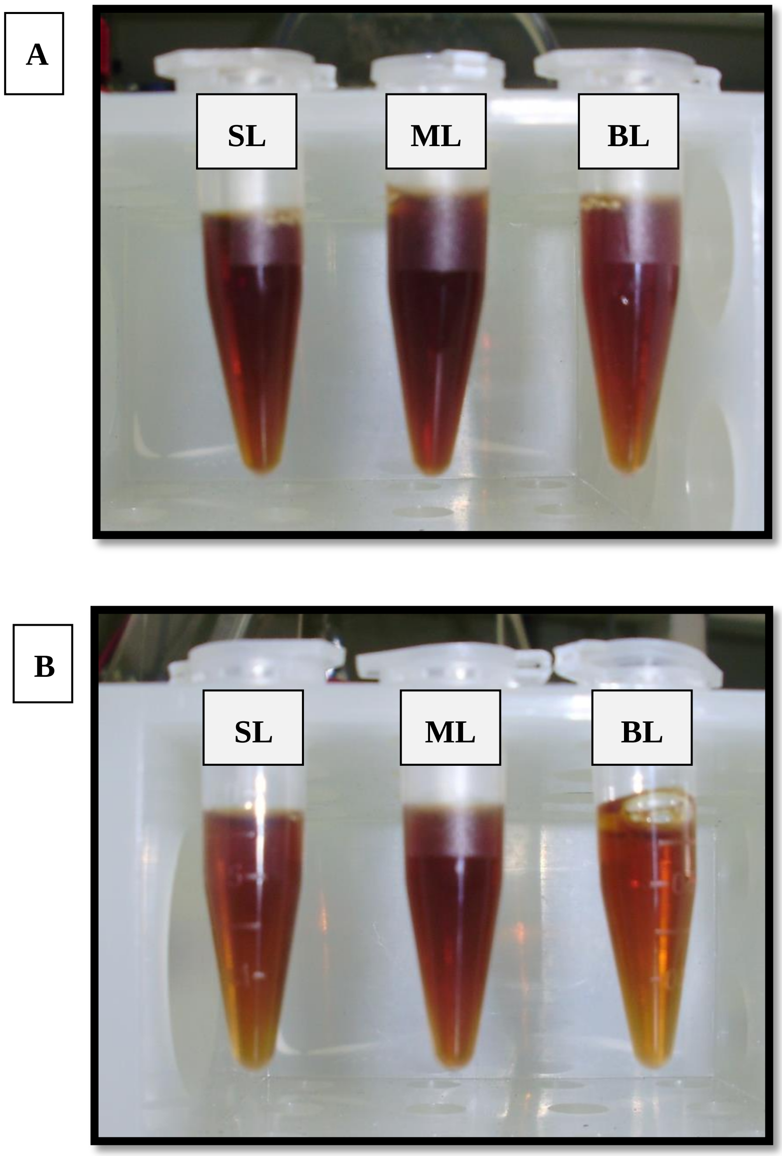


Figure 4.3 Comparison of the colour of *F. deltoidea* crude extracts pre- and post-PVPP treatment

Picture A showed the original colour of SL, ML and BL crude extracts. Picture B showed the *F. deltoidea* extracts after being treated with PVPP. Slight changes of the extract's colour were observed after incubation with PVPP. Originally the extracts possesses dark brown colour. The colour of the extracts becomes lighter after being treated with PVPP.

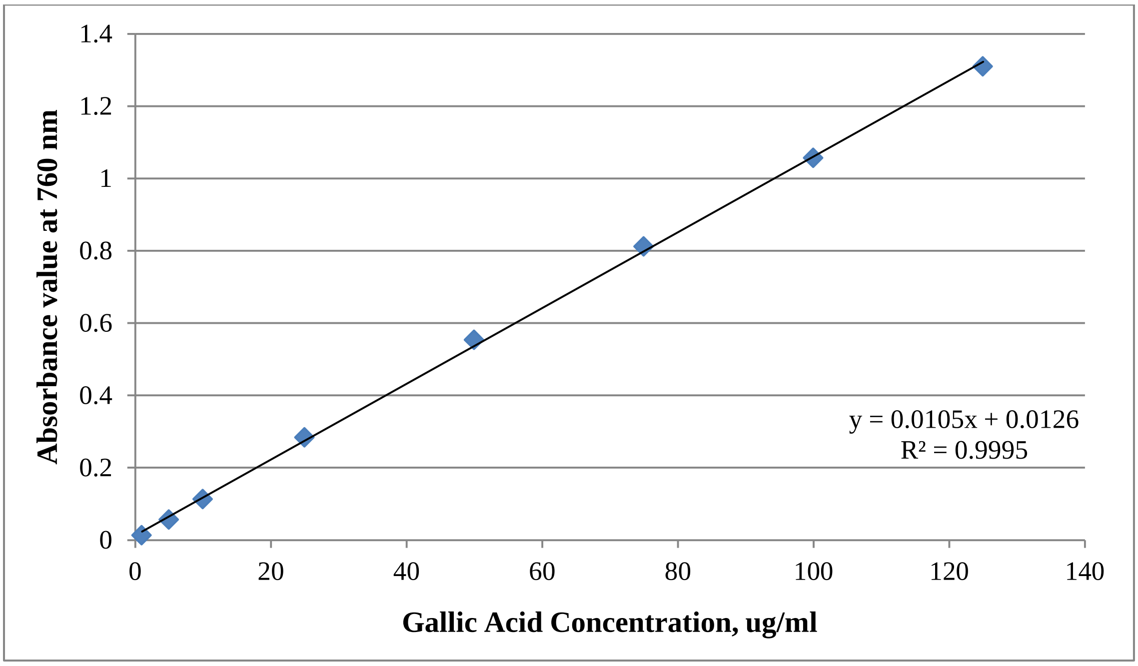


Figure 4.4 Gallic acid standard curve

Gallic acid was used as a reference for total phenolic content assay. Total phenolic content in samples were determined from the equation y = 0.0105x + 0.0126, which generated from the standard curve with R^2 value 0.9995.

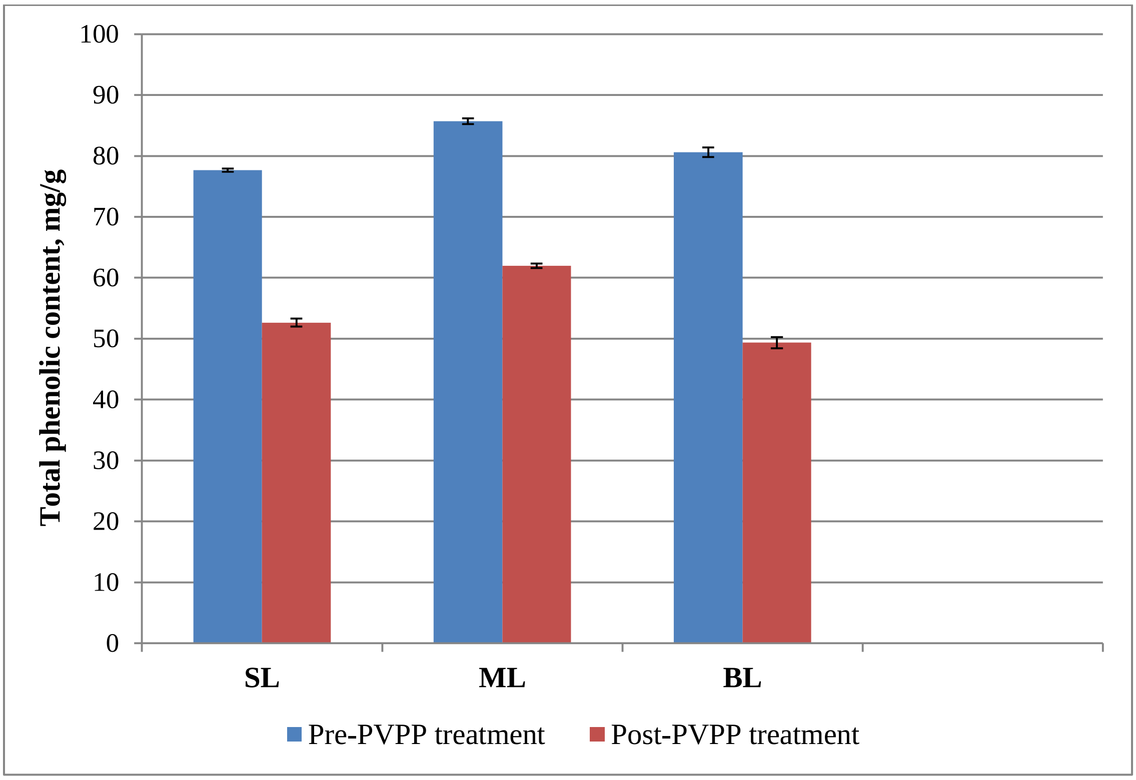


Figure 4.5 Comparison of total phenolic content in pre- and post-PVPP treatment extracts

As shown in the graph, ML extracts contains slightly higher phenolic content among the three varieties. All the extracts prior to PVPP treatment have higher total phenolic content compared to post-PVPP treatment extracts.

4.3 Comparison of Laemmli Buffer System and Tricine-SDS-PAGE System

Protein extracts were resolved in 18 % gel with Laemmli buffer system and 16 % gel with Tricine-SDS-PAGE system. Their gel profiles were shown in Figure 4.6. Resolutions of the protein bands were compared among these two buffer system. Sharp protein bands (red circle) were observed at high MW region in the gel resolved by Laemmli buffer system. However in the region below 6.5 kDa (green circle), diffused and blur bands were observed. In the SDS gel resolved by Tricine-SDS-PAGE (as shown in Figure 4.6 (b), (d) and (f)), sharp bands were observed in both the high and low MW region. Based on the results, Tricine-SDS-PAGE system was chosen for subsequent analysis.

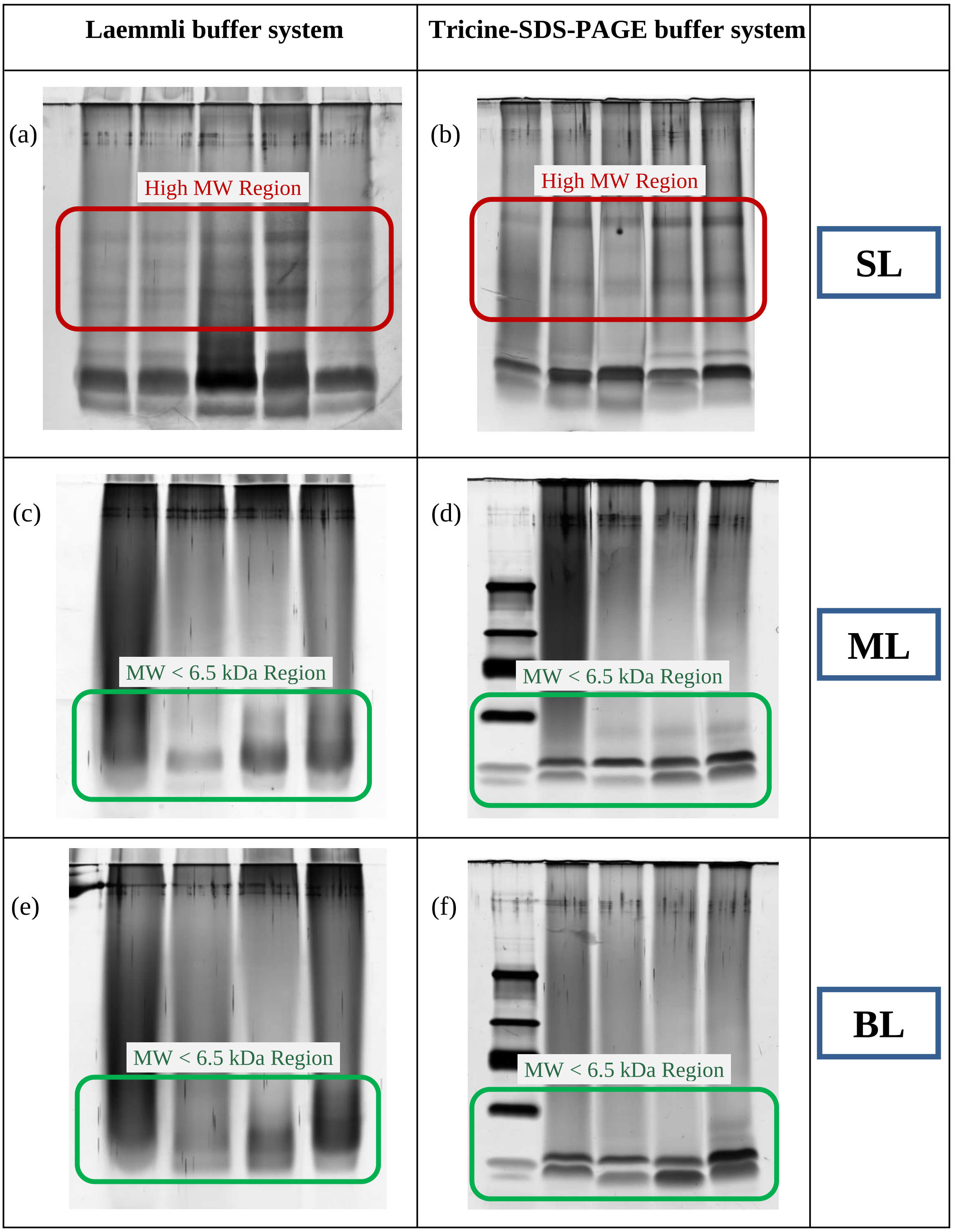


Figure 4.6 Comparison of protein profiles using Laemmli and Tricine-SDS-PAGE systems

On the left (a), (c) and (e) showed samples resolved by Laemmli system in 18 % gel, while on the right (b), (d) and (f) are samples resolved by Tricine-SDS-PAGE system in 16 % gel. Red circle indicated high MW region in the protein profile, whereas green circle highlighted the region containing protein bands below 6.5 kDa.

4.4 Comparative Evaluation of the Four Precipitation Methods

4.4.1 Protein recovery

Proteins in *Ficus* leaves aqueous extracts were extracted using four different precipitation methods. They were 90 % ammonium sulfate precipitation (Method 1), acetone cold precipitation (Method 2), TCA/acetone precipitation (Method 3) and 90 % ammonium sulfate precipitation followed by acetone/β-mercaptoethanol precipitation (Method 4). Dry weight of the crude protein was measured after freeze-dry process. Crude protein yielded from the extracts following four precipitation methods were shown in Table 4.1. Protein content in crude protein extracts were quantified using BCA® Protein Assay Kit. BSA standard curve (Figure 4.7) was created using known protein concentration and its blank-corrected absorbance value (Table 8.2 - Appendix). Protein concentrations were determined from the equation obtained from the standard curve, which was $y = 0.001x$. Protein content in 1 mg crude protein extract and the purity percentage of the extracts are shown in Table 4.2. Comparison of the crude protein yield from different protein precipitation and their purity are presented in bar chart shown in Figure 4.8.

Among the four methods, Method 2 gave the greatest crude protein yield with an intermediate purity of the extracts. Crude protein yield of Method 1 and 3 were intermediate. However, samples from Method 1 have the highest purity, while samples from Method 3 have the lowest purity among them all. Method 4 gave the lowest yield, but their crude protein showed considerably higher purity. Besides that, freeze-dried extracts obtained from Method 1 and 2 possesses dark brown colour. Whereas, light yellowish-brown powder extracts were obtained from Method 3 and 4.

Table 4.1 Crude protein yielded from four different precipitation methods

Sample	Precipitation method	Crude Protein Yield (mg/g crude extract)
SL	Method 1 (SL1)	53.27 ± 5.91
	Method 2 (SL2)	360.47 ± 67.33
	Method 3 (SL3)	46.57 ± 7.15
	Method 4 (SL4)	21.80 ± 2.36
ML	Method 1 (ML1)	48.47 ± 6.01
	Method 2 (ML2)	424.43 ± 45.68
	Method 3 (ML3)	67.13 ± 6.22
	Method 4 (ML4)	18.20 ± 1.91
BL	Method 1 (BL1)	49.13 ± 1.33
	Method 2 (BL2)	279.63 ± 48.75
	Method 3 (BL3)	69.60 ± 1.51
	Method 4 (BL4)	18.60 ± 2.03

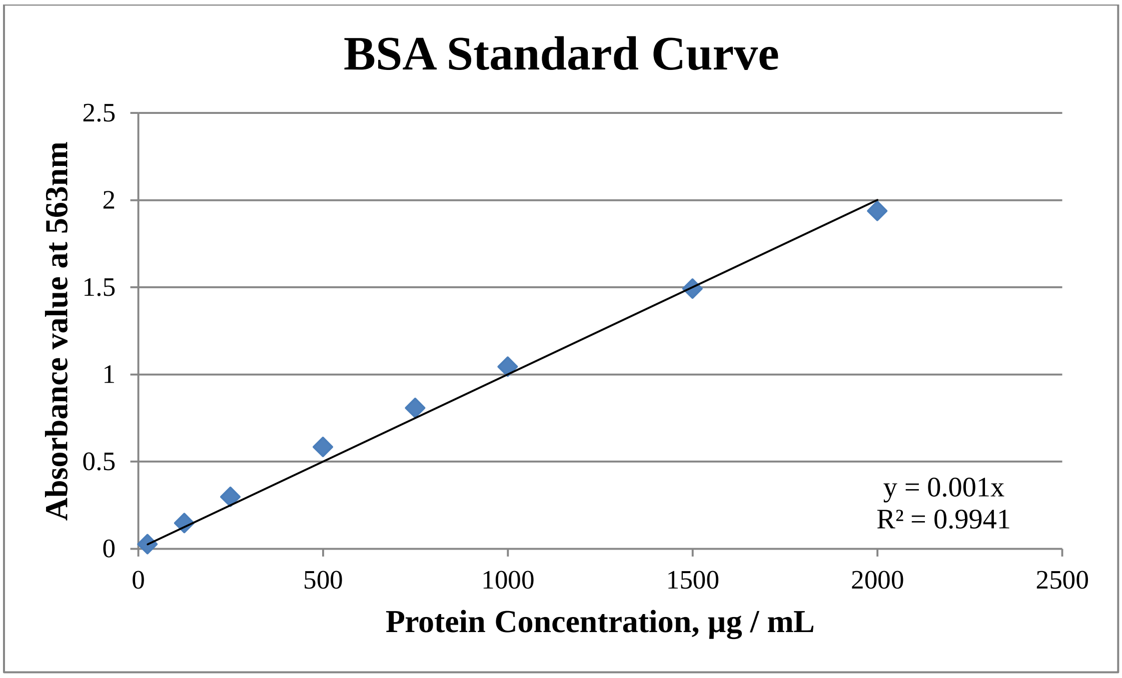


Figure 4.7 BSA Standard Curve

Known protein concentrations were plotted against their blank-corrected absorbance value to create a standard curve. The equation for the standard curve is y = 0.001x, with R^2 value 0.9941. Protein concentration of the extracts was determined from the standard equation.

Table 4.2 Protein content and purity of the protein extracts

Sample	Protein content in 1 mg crude protein extract, µg	Percentage of protein content in crude protein extract, %
SL1	838.66 ± 10.49	83.87 ± 1.05
SL2	229.79 ± 5.79	22.98 ± 0.58
SL3	95.81 ± 1.89	9.58 ± 0.19
SL4	479.65 ± 8.30	47.97 ± 0.83
ML1	906.62 ± 18.93	90.66 ± 1.89
ML2	351.57 ± 15.68	35.16 ± 1.57
ML3	125.32 ± 7.15	12.53 ± 0.72
ML4	315.98 ± 4.84	31.60 ± 0.48
BL1	687.74 ± 17.88	68.77 ± 1.79
BL2	203.64 ± 2.51	20.36 ± 0.25
BL3	112.02 ± 9.39	11.20 ± 0.94
BL4	289.55 ± 9.22	28.96 ± 0.92

Percentage of protein content in crude protein extract (purity of crude protein extract) was calculated following the equation stated below:

$$\text{Percentage of protein content in crude protein extract} = \frac{\text{Protein content in 1 mg crude protein extract (µg)}}{\text{1000 µg crude protein extract}} \times 100\ \%$$

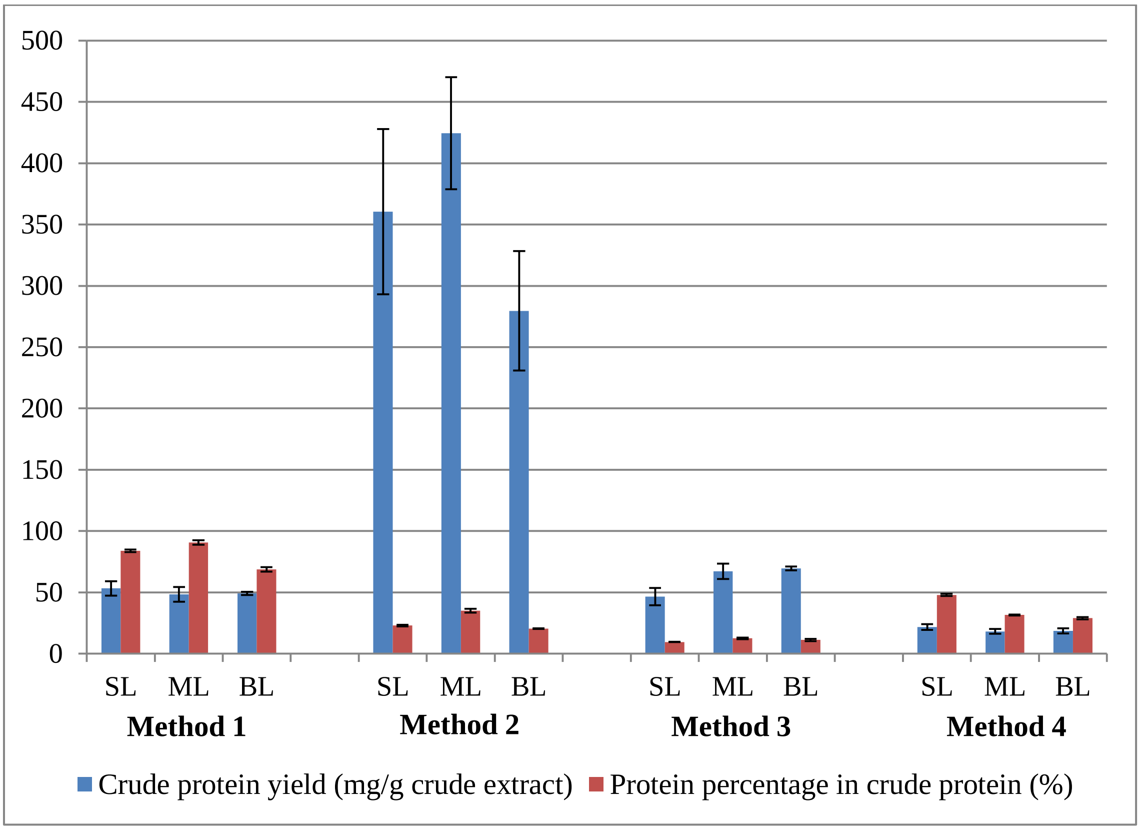


Figure 4.8 Comparison of crude protein yield and their purity

The four protein precipitation methods involved were 90 % ammonium sulfate precipitation (Method 1), acetone cold precipitation (Method 2), TCA/acetone precipitation (Method 3) and 90 % ammonium sulfate precipitation followed by acetone/β-mercaptoethanol precipitation (Method 4). Method 2 gave the highest crude protein yield, while Method 1 showed the highest purity. Among the four, Method 4 has the lowest yield with a considerably higher purity of the extracts.

4.4.2 Comparison of Tricine-SDS-PAGE profiles of *F. deltodea* samples

Proteins extracted from the four methods of precipitation were analysed by Tricine-SDS-PAGE buffer system. For samples obtained from Method 1 (SL1, ML1 and BL1) and Method 2 (SL2, ML2 and BL2), 40 µg of protein were loaded onto the wells. For samples obtained from Method 3 (SL3, ML3 and BL3) and Method 4 (SL4, ML4 and BL4), 20 µg of protein were loaded onto the wells. They were resolved in 16 % tricine gel and visualized by silver staining. Figure 4.9, 4.10 and 4.11 showed the Tricine-SDS-PAGE profiles of SL, ML and BL proteins obtained from the four precipitation protocols.

Tricine-SDS-PAGE protein profiles of SL leaves protein extracts obtained from four different precipitation methods are shown in Figure 4.9. Smearing background and fade bands were observed in SL1 (Lane 1). Despite the banding pattern is similar among the four, it was observed that bands at MW 5.97 kDa and 3.82 kDa are more prominent in SL4 (Lane 5) than others. Also, SL4 (Lane 5) has sharper bands compared to the others. Figure 4.10 showed the Tricine-SDS-PAGE protein profiles of ML leaves protein extracts obtained from four different precipitation methods. ML1 (Lane 1) has dark smearing background. The banding pattern did not differ much among the four profile of ML, though an additional band was observed in ML4 (Lane 5) at MW 3.66 kDa. Figure 4.11 revealed the Tricine-SDS-PAGE protein profiles of BL leaves protein extracts obtained from four different precipitation methods. BL1 (Lane 2) and BL3 (Lane 4) have identical profile, whereas BL2 (Lane 3) and BL4 (Lane 5) have similar profile. Two bands at MW 4.85 kDa and 3.74 kDa were observed in both the profile of BL2 (Lane 3) and BL4 (Lane 5), however their intensities are higher in BL4 (Lane 5).

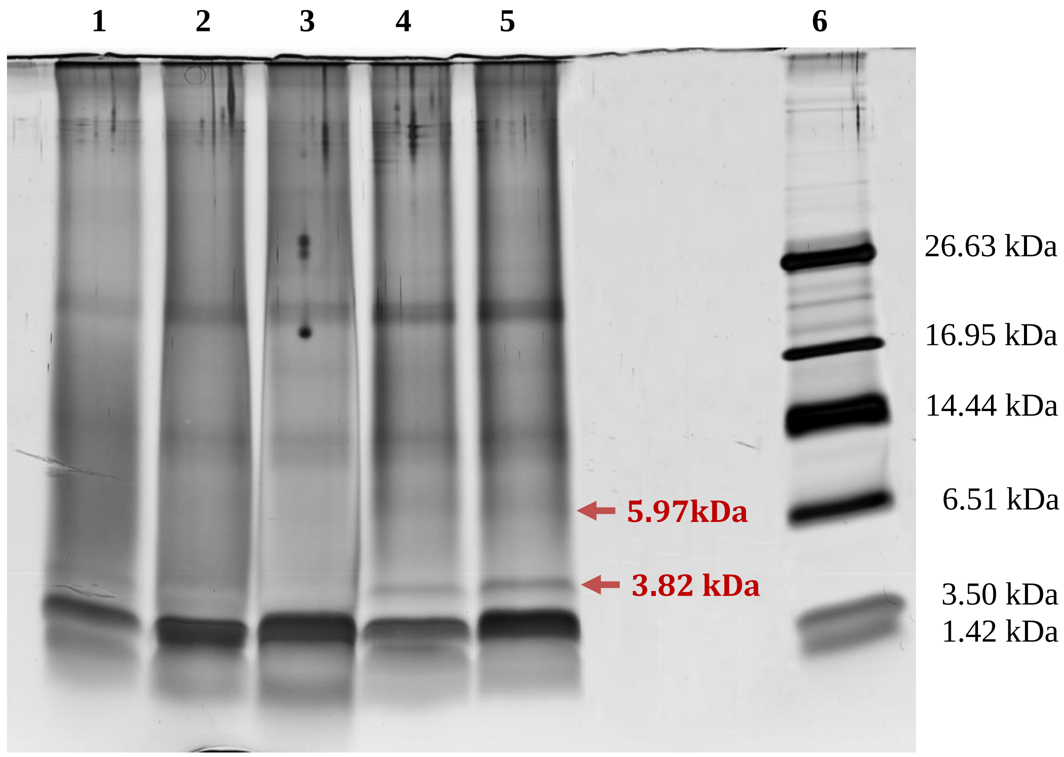


Figure 4.9 Comparison of Tricine-SDS-PAGE protein profiles of SL leaves protein extracts

Proteins in SL leaves aqueous extracts were extracted using four different precipitation methods and resolved by SDS-PAGE. Polypeptide standard from Bio-rad (Lane 6) was used as a reference for protein MW. Similar banding pattern was observed for all the four SL samples. Band 5.97 kDa and 3.82 kDa indicated the two bands that are more prominent in SL4.

Lane 1: Method 1 (SL1)

Lane 2: Method 2 (SL2)

Lane 3: Method 3 (SL3)

Lane 4 and 5: Method 4 (SL4)

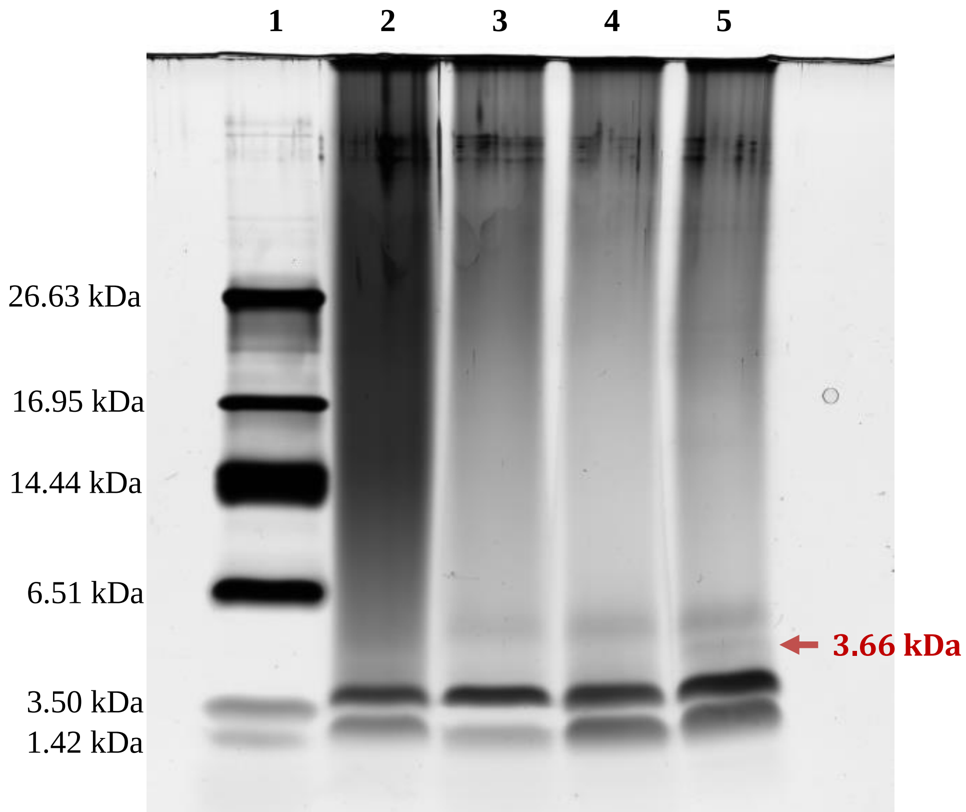


Figure 4.10 Comparison of Tricine-SDS-PAGE protein profiles of ML leaves protein extracts

Proteins in ML leaves aqueous extracts were extracted using four different precipitation methods and resolved by SDS-PAGE. Polypeptide standard from Bio-rad (Lane 1) was used as a reference for protein MW. It was observed that an additional band was observed in ML4 profile at 3.66 kDa.

Lane 2: Method 1 (ML1)

Lane 3: Method 2 (ML2)

Lane 5: Method 3 (ML3)

Lane 5: Method 4 (ML4)

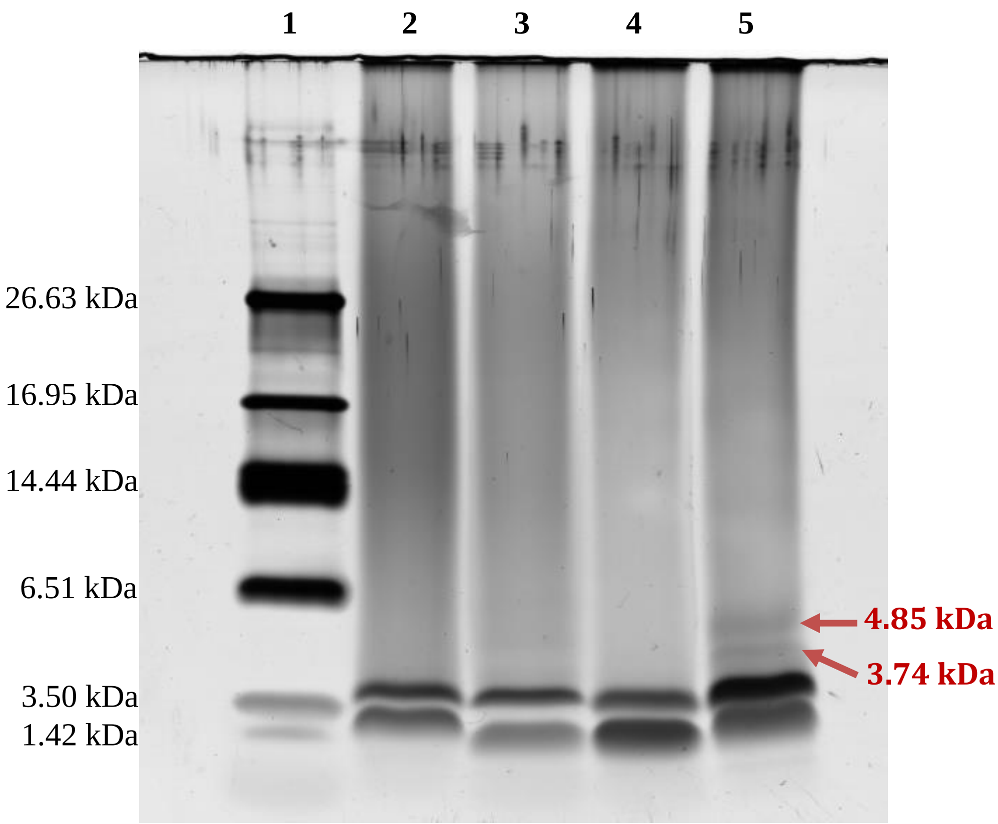


Figure 4.11 Comparison of Tricine-SDS-PAGE protein profiles of BL leaves protein extracts

Proteins in BL leaves aqueous extracts were extracted using four different precipitation methods and resolved by SDS-PAGE. Polypeptide standard from Bio-rad (Lane 1) was used as a reference for protein MW. The profile of BL1 and BL3 were identical, whereas the profile for BL2 and BL4 were similar. Band 4.85 kDa and 3.74 kDa indicated the two bands that are more prominent in the profile of BL4.

Lane 2: Method 1 (BL1)

Lane 3: Method 2 (BL2)

Lane 5: Method 3 (BL3)

Lane 5: Method 4 (BL4)

4.5 Protein Profiling of *F. deltoidea* Aqueous Extracts using SDS-PAGE

SDS-PAGE protein profiles of three varieties of *F. deltoidea* were presented in Figure 4.12. A calibration curve for MW estimation (Figure 8.1 in Appendix) was plotted using the MW of standard protein and their calculated R_f values. MW of protein bands were determined using the equation obtained from calibration curve. Table 4.3 listed the estimated MW of protein bands that are present in the profiles of SL, ML and BL.

Tricine-SDS-PAGE analysis of the leaves aqueous extracts of three varieties of *F. deltoidea* revealed the presence of proteins in the range from 101 kDa down to 1.42 kDa. Generally, the profiles can be categorized into three regions, Region A, Region B and Region C (as shown in Figure 4.12). Region A consists of protein bands with MW higher than 26.6 kDa. Region B consists of protein bands ranged from 26.6 kDa down to 6.5 kDa, while region C consists of low MW proteins which are less than 6.5 kDa. In SL profile, protein bands were observed in Region A, B and C. In Region A, a group of estimated three to four thin and streaking bands were observed. Those bands were not well resolved. Six protein bands were observed in Region B, whereas four protein bands were observed in Region C. Among the protein bands in Region B and C, bands at 33.66 ± 0.44 kDa, 15.89 ± 0.46 kDa and 7.48 ± 0.17 kDa are very fade. In the profiles of ML and BL, protein bands were observed only in Region A and C. Similar to SL profile, thin and streaking protein bands were observed in Region A in both ML and BL profiles. Four protein bands were observed in Region C of both profiles. All the observed bands were listed in Table 4.3.

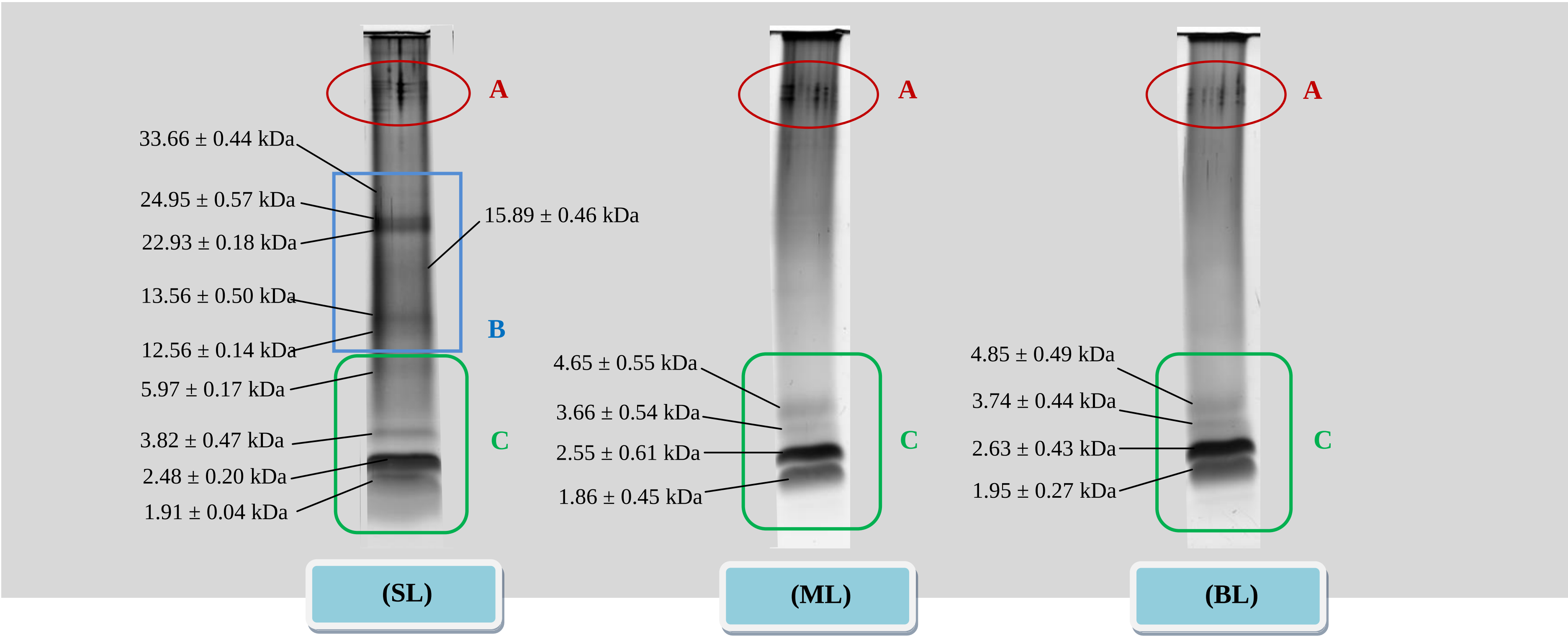


Figure 4.12 Comparison of SDS-PAGE protein profiles of three varieties of *F. deltoidea* leaves aqueous extracts (SL, ML and BL)

The profiles were categorized into three regions. Region A which circled in red showed the MW region higher than 26.6 kDa. Region B which highlighted in blue box showed the MW region ranged from 26.6 kDa down to 6.5 kDa. Region C which circled in green indicated the MW region less than 6.5 kDa.

Table 4.3 Estimated MW of protein bands observed in the SDS-PAGE profiles of SL, ML and BL

Region	**Molecular Weight (MW), kDa**		
	SL	**ML**	**BL**
B	33.66 ± 0.44	-	-
B	24.95 ± 0.57	-	-
B	22.93 ± 0.18	-	-
B	15.89 ± 0.46	-	-
B	13.56 ± 0.50	-	-
B	12.56 ± 0.14	-	-
C	5.97 ± 0.17	4.65 ± 0.55	4.85 ± 0.49
C	3.82 ± 0.47	3.66 ± 0.54	3.74 ± 0.44
C	2.48 ± 0.20	2.55 ± 0.61	2.63 ± 0.43
C	1.91 ± 0.04	1.86 ± 0.45	1.95 ± 0.27

4.6 Protein profiling of *F. deltoidea* Aqueous Extracts using 2-D Gel Electrophoresis

Protein extracts of SL, ML and BL obtained from Method 4 were further analysed by 2-D gel electrophoresis. Figure 4.13- 4.15 showed the proteome of SL, ML and BL respectively. Gels were analysed using Melanie 7.0 (GeneBio). It was found that all protein spots in the proteome were mainly focused at two regions, within the range of 53 kDa to 78 kDa, and 6.5 kDa to 28 kDa. Therefore, the region of 53 kDa to 78 kDa is marked as Region X, whereas the region of 6.5 kDa to 28 kDa is marked as Region Y. Furthermore, it was observed that protein bands with MW less than 6.5 kDa were missing in all the 2-D proteome of SL, ML and BL.

A total of forty-five spots were detected in the proteome of SL by Melanie analysis (Figure 4.13). Thirty-three spots were resolved in Region X, and twelve spots were resolved in Region Y. All proteins in Region Y have a pI value lower than seven. Figure 4.16 and 4.17 showed a closer look at the protein spots present in SL proteome.

In the proteome of Ml and BL, protein spots were only found at Region X. No protein spots observed in Region Y. In Region X, twenty-four and nine spots were detected in the proteome of ML and BL respectively (Figure 4.14 and 4.15). Besides that, vertical streaking backgrounds are observed towards the acidic side of the gels. Figure 4.18 and 4.19 showed a closer look at the protein spots detected in ML and BL proteome respectively.

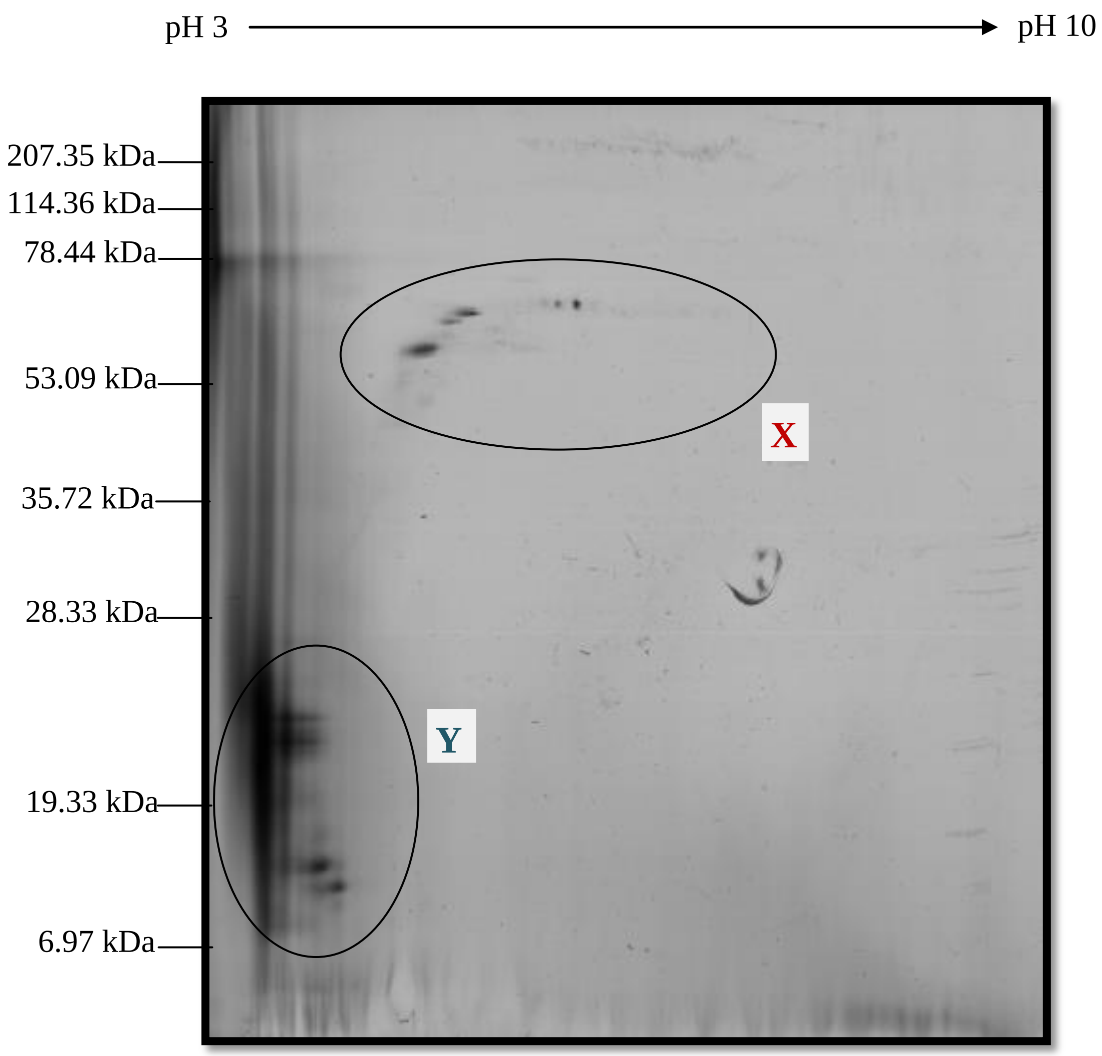


Figure 4.13 2-DE protein profile of leaves aqueous extracts of SL

200 µg of protein obtained from Method 4 was loaded on the gel. Proteins were separated on pH 3 – 10 IPG strip and 10 – 18 % gradient gel. Protein spots are mainly focus on Region X and Y as indicated above. Region X lies between the range of MW 53 kDa - 78 kDa, while Region Y lies between the range of MW 6.5 kDa to 28 kD. Protein markers were separated together with protein sample on the gel and their positions are as shown on the left.

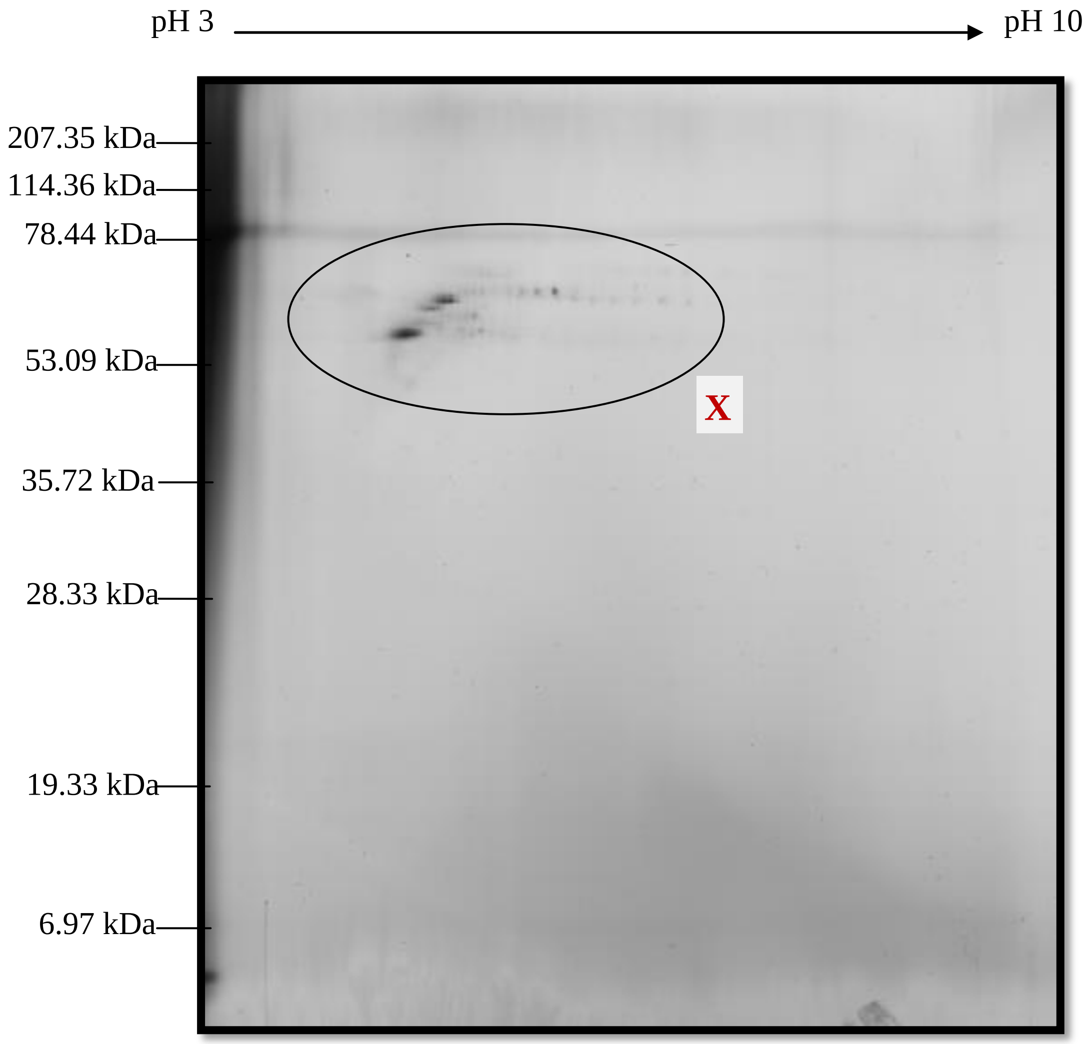


Figure 4.14 2-DE protein profile of leaves aqueous extracts of ML

200 µg of protein obtained from Method 4 was loaded on the gel. Proteins were separated on pH 3 – 10 IPG strip and 10 – 18 % gradient gel. Protein spots are mainly focus on Region X as highlighted above. Region X lies between the MW range of 53 kDa - 78 kDa. Protein markers were separated together with protein sample on the gel and their positions are as shown on the left.

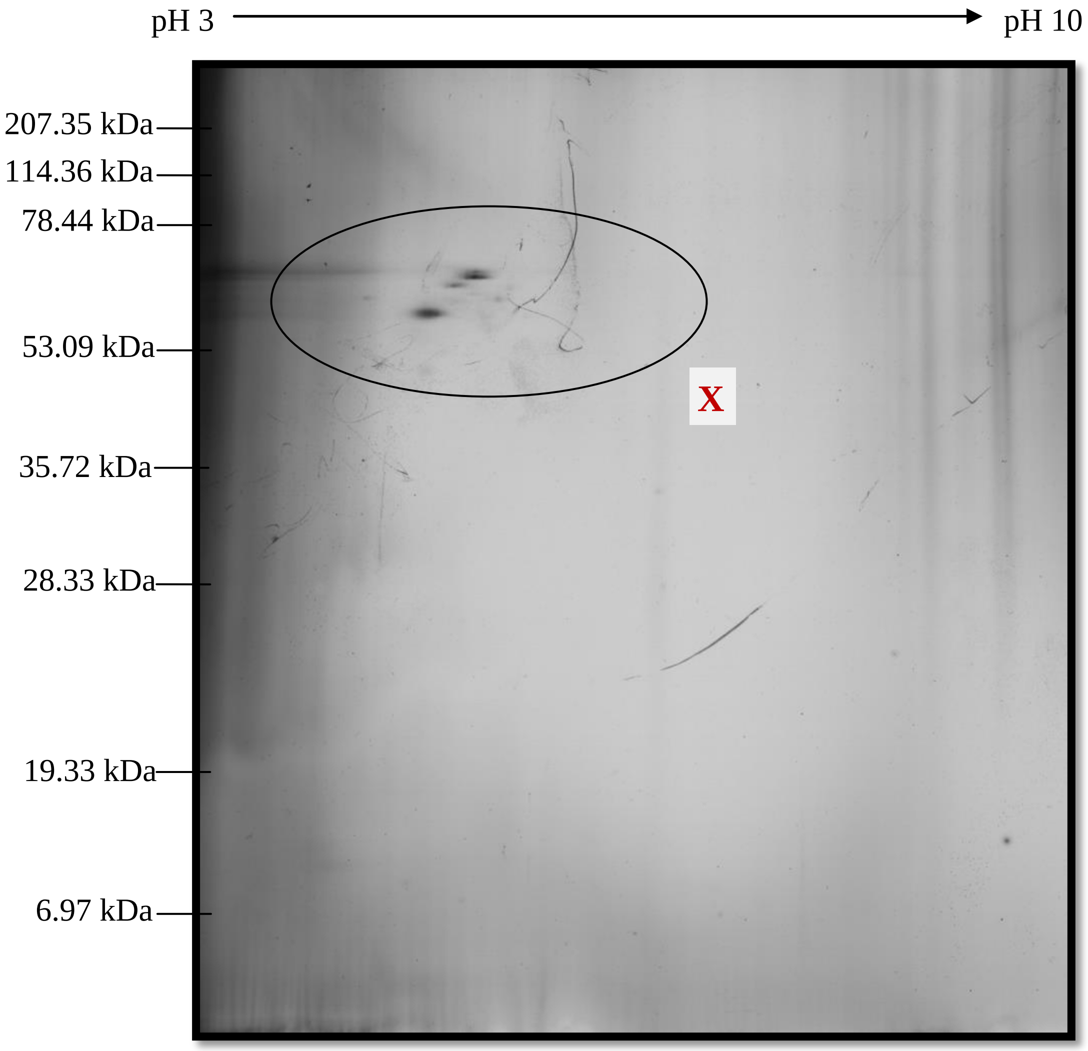


Figure 4.15 2-DE protein profile of leaves aqueous extracts of BL

200 µg of protein obtained from Method 4 was loaded on the gel. Proteins were separated on pH 3 – 10 IPG strip and 10 – 18 % gradient gel. Protein spots are mainly focus on Region X as highlighted above. Region X lies between the MW range of 53 kDa - 78 kDa. Protein markers were separated together with protein sample on the gel and their positions are as shown on the left.

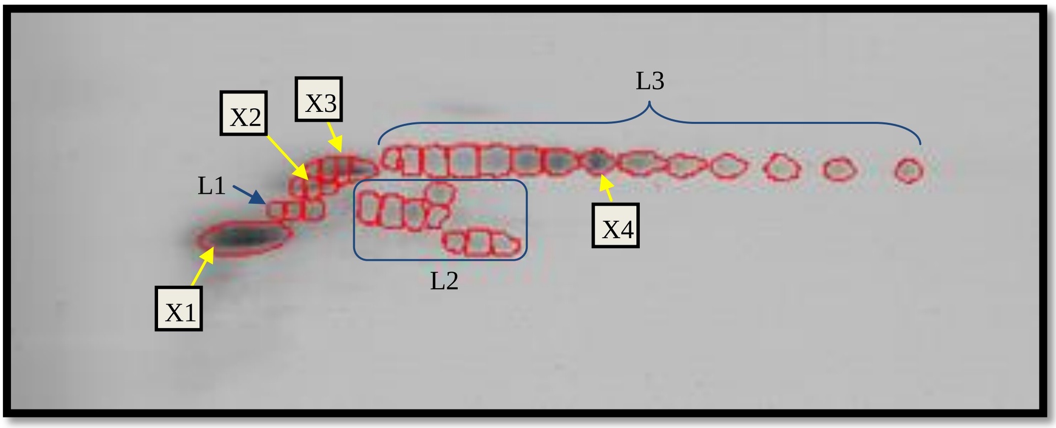


Figure 4.16 Region X in SL 2-DE protein profile

This region lies between the MW range of 53 – 78 kDa. A total of 33 spots were detected in this region by Melanie analysis. Spot X1, X2, X3 and X4 were further analysed by MALDI-TOF-TOF.

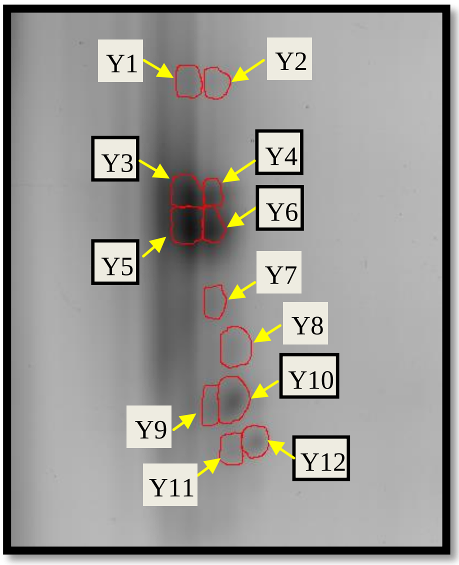


Figure 4.17 Region Y in SL 2-DE protein profile

This region lies between the MW range of 6.5 - 28 kDa. 12 spots were detected in this region by Melanie analysis. Spot Y3, Y4, Y5, Y6, Y10 and Y12 were further analysed by MALDI-TOF-TOF.

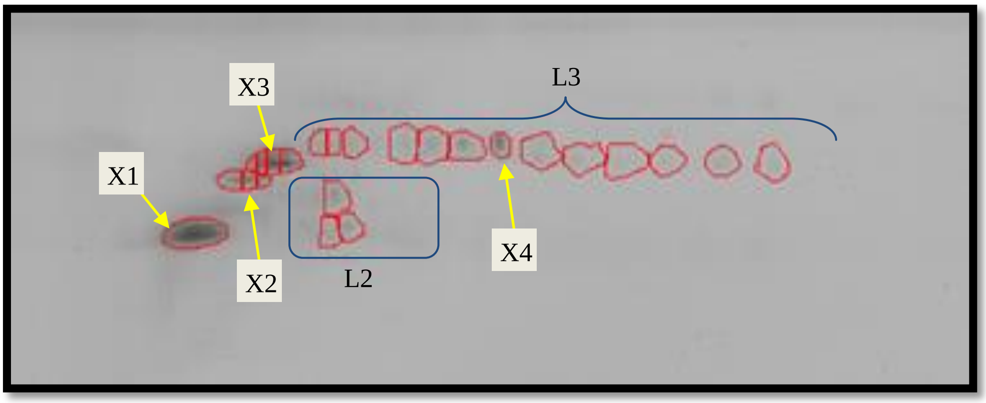


Figure 4.18 Region X in ML 2-DE protein profile

This region lies between the range of MW 53 – 78 kDa. A total of 24 spots were detected in this region. X1, X2 and X3 are common protein spots in the proteome of SL, ML and BL, whereas X4 is only present in SL and ML proteome.

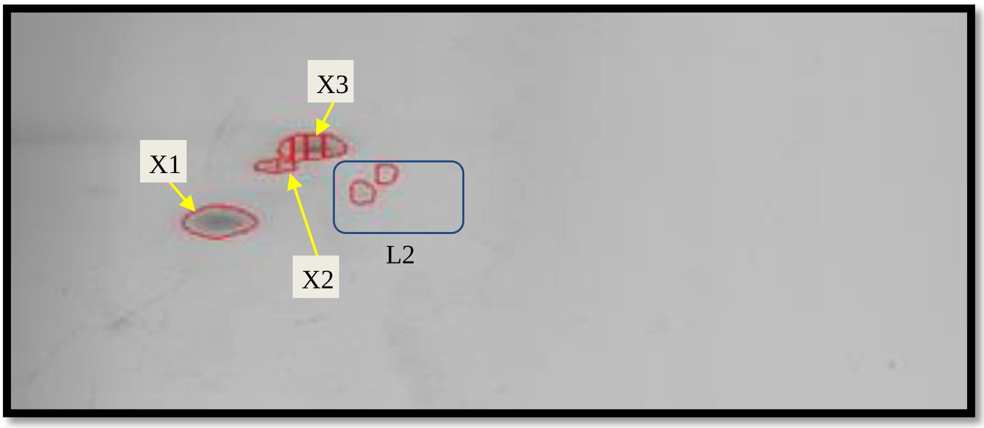


Figure 4.19 Region X in BL 2-DE protein profile

This region lies between the range of MW 53 – 78 kDa. A total of 9 spots were detected in this region. X1, X2 and X3 are common protein spots in the proteome of SL, ML and BL.

4.7 Identification and Characterization of the Protein Spots

MALDI analyses of all the spots were difficult due to insufficient amount of protein and challenges in getting reproducible gel. Hence, only certain spots with higher intensity were selected to proceed with MALDI-TOF-TOF analysis. Spots selected for MALDI-TOF-TOF analysis were: X1, X2, X3, X4, Y3, Y4, Y5, Y6, Y10 and Y12. Among the protein spots in Region X, Spot X1, X2 and X3 were selected as they are present in all the three proteome. Another high intensity protein spot selected was X4. It is present in both the proteome of SL and ML. Protein spot Y3, Y4, Y5, Y6, Y10 and Y12 were among those proteins unique to SL proteome. They were searched against *Ficus* species database in NCBI database. Results obtained from MALDI analysis were presented in Table 4.4

Table 4.4 Identified proteins from MALDI-TOF-TOF analysis

Spot Number	Protein name [species]	Protein Abbreviation	Accession Number / Gene Index (gi) / Enzyme Code	MW / pI	Protein score	Sequence Coverage (%)
X1	Photosystem II reaction center protein I [*Leptosira terrestris*]	PSBI_LEPTE	A6YG99 / 5383786	3911 / 9.98	13	85
X2	Thioredoxin M-type, chloroplast precursor (Trx-M) [*Zea mays* (Maize)]	TXM_MAIZE	Q41864 / 542254	18061 / 8.70	14	22
X3	Glutamyl-tRNA reductase 2, chloroplast precursor (GluTR) [*Arabidopsis thaliana* (Mouse-ear cress)]	HEM12_ARATH	P49294 / 837528 [EC 1.2.1.70]	58269 / 8.82	25	23
X4	Sucrose synthase (Sucrose-UDP glucosyltransferase) [*Solanum tuberosum* (Potato)]	SUS2_SOLTU	P49039 / 1351137 [EC 2.4.1.13]	92519 / 5.98	15	8
Y3	Ribulose-1,5-biphosphate carboxylase/oxygenase large subunit [*Ficus deltoidea* var. *intermedia*]	G3CKP0_9ROSA	G3CKP0 / 345296489	47696.1 / 6	13	85.641

Table 4.4 (continued) Identified proteins from MALDI-TOF-TOF analysis

Y4	Ribulose-1,5-biphosphate carboxylase/oxygenase large subunit [*Ficus deltoidea* var. *intermedia*]	G3CKP0_9ROSA	G3CKP0 / 345296489	47696.1 / 6	13	83.889
Y5	Ribulose-1,5-biphosphate carboxylase/oxygenase large subunit [*Ficus deltoidea* var. *intermedia*]	G3CKP0_9ROSA	G3CKP0 / 345296489	47696.1 / 6	13	85.307
Y6	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Ficus cordata]	CAJ86904.1	CAJ86904 / 125991597	51960.3 / 6	38	88.388
Y10	ATP synthase CF-1 alpha subunit [*Ficus paludica*]	D0UFQ6_9ROSA	D0UFQ6 / 261826038	4170.3 / 6.07	29	54.48
Y12	ATP synthase CF1 alpha subunit [*Ficus variegata* Blume, 1825]	D0UFP7_9ROSA	D0UFP7 / 261826020	4455.4 / 5.05	28	46.519

4.8 SELDI-TOF Profiling

4.8.1 Protein profiling of *F. deltoidea* leaves aqueous extracts using SELDI-TOF

SELDI-TOF profiling was carried out using ProteinChip® SELDI System at Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya. Optimization on the laser energy used was carried out beforehand. The laser energy was optimized at 1200 nJ. SELDI-TOF profiling on ProteinChip H50 was performed on SL, ML and BL protein extracts obtained from four different precipitation methods. Each sample was analysed in triplicate on ProteinChips H50. Peaks detection, normalization and clustering were performed using ProteinChip Data Manager Software 3.5. A blank control has been done by replacing the sample with Milli-Q water. SELDI-TOF spectrum for blank control is shown in Figure 4.20. Some peaks were observed at *m/z* 100 – 1,700 in the spectrum of blank control, therefore analyses of sample's spectrum below *m/z* 2,000 were excluded. No peak was observed at *m/z* value over 15,000 from earlier optimization. Therefore, spectral analyses were limited to the range of *m/z* 2,000 – 15,000. Representative SELDI-TOF spectra of SL, ML and BL extracts obtained from four different precipitation methods on ProteinChip H50 are shown in Figure 4.21, 4.25 and 4.29 respectively. Peaks detected in each spectrum were presented in Table 4.5.

Among the four spectrum profiles of SL shown in Figure 4.21, SL1 has the most peak detected. They were peaks with *m/z* 3744, 7484 and 7645. The spectrum profiles of SL2 and SL4 have the least peak, there are only one peak was detected at *m/z* ~7480. However, several humps were observed in the spectrum of SL4. A huge hump was observed around *m/z* 4000 to 5800, where there were two undetected peaks observed at *m/z* 4000 and 5320. Another 4 humps were seen at *m/z* 6799, 8009, 10719 and 13614 in the spectrum of SL4 and there was a small peak observed on each bump. Based on peak detection, peaks with *m/z* 4000, 5320 and 6799 might be doubly charged ions of peaks with *m/z* 8009, 10719 and 13614 respectively. The spectrum profile of SL3 has 2 peaks detected at *m/z* 3743 and 7478. As shown in Figure 4.22, peak with *m/z* 7480 was constantly present in the spectrum profiles of SL obtained from all the four methods. However, EDM statistical analysis revealed their peak intensities significantly differ among each other. Figure 4.23 revealed the cluster report of protein peak with *m/z* 7480 with a detection of *P*-value at 0.024. The intensity of protein peak with *m/z* 7840 in SL1 was much higher than the others, followed by SL4, SL3 and SL2 (as shown in Figure 4.24). Peak with *m/z* 3744 was present in the spectrum profiles of SL1 and SL3, whereas peak with *m/z* 7645 was only present in the spectrum profile of SL1.

Spectrum profiles of ML sample from the four precipitation methods are shown in Figure 4.25. ML1 and ML3 have the same spectrum profiles where both of the profiles have 4 peaks detected at *m/z* 3744, 3825, 7480 and 7640. Two peaks were detected in the spectrum profile of ML2. They were peaks with *m/z* 3744 and 7480. The spectrum profile of ML4 has the least peak. Only one peak was detected at *m/z* 7480. Figure 4.26 showed that peak with *m/z* 3744 was detected in the spectrum profiles of ML1, ML2 and ML3. In the spectrum of ML4, peak with *m/z* 3744 was observed but not detected as it was present below the detection limit. Peak with *m/z* 3825 was only present in the spectrum profiles of ML1 and ML3, while peak with *m/z* 7480 was present and detected in all the four spectrum profiles of ML. Peak with *m/z* 7640 was observed in all the four spectrum profiles, but it was only detected in ML1 and ML3. EDM statistical analysis by comparing the intensities of peak clusters with *m/z* 3744, 7480 and 7640 reports a detection of *P*-value at 0.024, 0.019 and 0.016 respectively (as shown in Figure 4.27). Overall, ML1 spectrum gave the highest peak intensity compared to the others (as shown in Figure 4.28).

Spectrum profiles of BL sample from the four precipitation methods are shown in Figure 4.29. Spectrum profile of BL1 and BL3 has the most peaks detected. They have four peaks detected at *m/z* 3744, 3825, 7480 and 7640. Two peaks were detected in the spectrum of BL2 and they were peaks with *m/z* 7480 and 7640. The spectrum profile of BL4 has only one peak detected at *m/z* 7480. Figure 4.30 showed that peak with *m/z* 3744 and 3825 were present in the spectrum profiles of BL1, BL4 and BL3, but were only detected in BL1 and BL3. Peak with *m/z* 7480 and 7640 were present in all the four spectrum of BL. However, peak with *m/z* 7640 was not automatically detected in the spectrum profile of BL4. EDM statistical analysis report revealed that the peak intensity of peak cluster with *m/z* 7480 and 7640 were significantly varied among the four BL sample, with a detection of *P*-value at 0.022 and 0.016 respectively (shown in Figure 4.31). Their intensities were significantly high in BL1 spectrum profile compared to the others (as shown in Figure 4.32).

Figure 4.20 SELDI-TOF spectrum of blank control

SELDI-TOF results are presented in a graph of ion abundance versus the *m/z* ratio. Peaks were automatically detected with the condition S/N > 5.0 intensity and valley depth using ProteinChip Data Manager Software 3.5. Six peaks with *m/z* less than 1687 were detected in the spectrum.

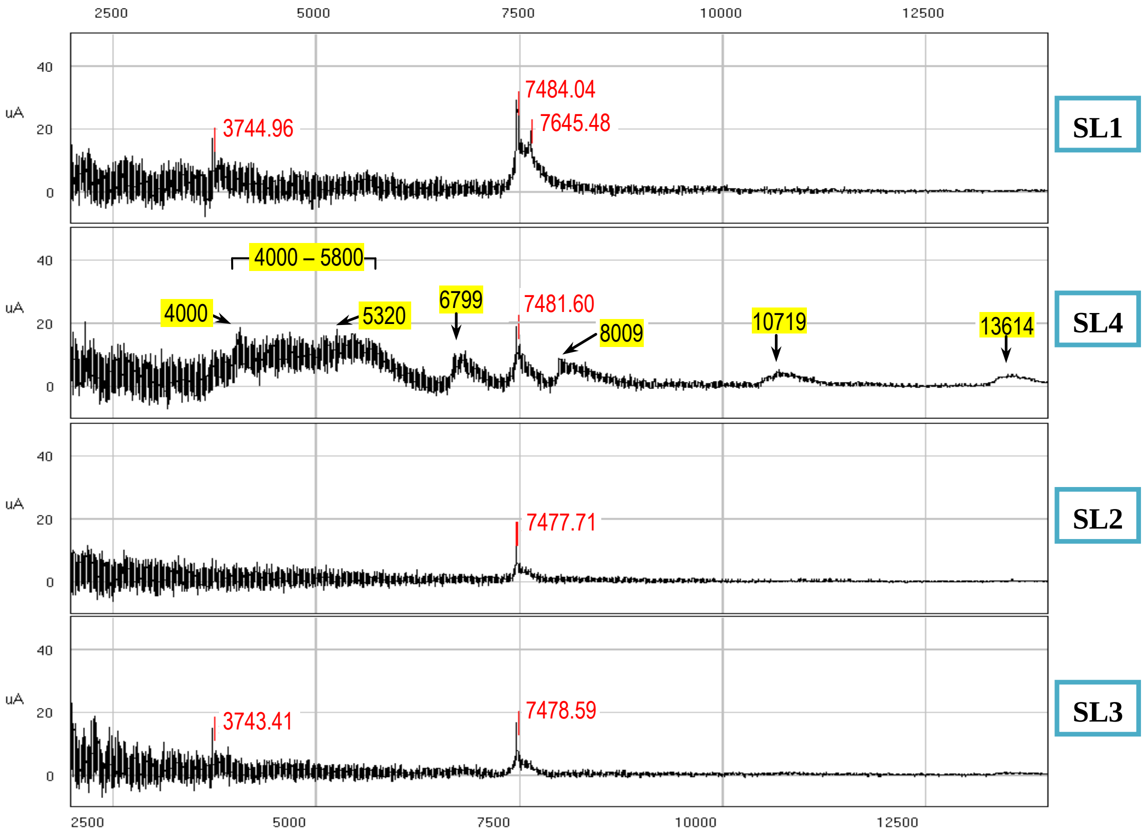


Figure 4.21 SELDI-TOF profiles of SL extracts

This figure showed the representative spectrum of SL sample obtained from Method 1 (SL1), Method 4 (SL4), Method 2 (SL2) and Method 3 (SL3). Peaks were automatically detected and normalized to total ion current intensity in 2 – 15 kDa using ProteinChip Data Manager Software 3.5 with the condition of 5.0 S/N valley depths and 5.0 S/N peak heights. The arrows indicated the manually detected peaks observed in the spectrum of SL4.

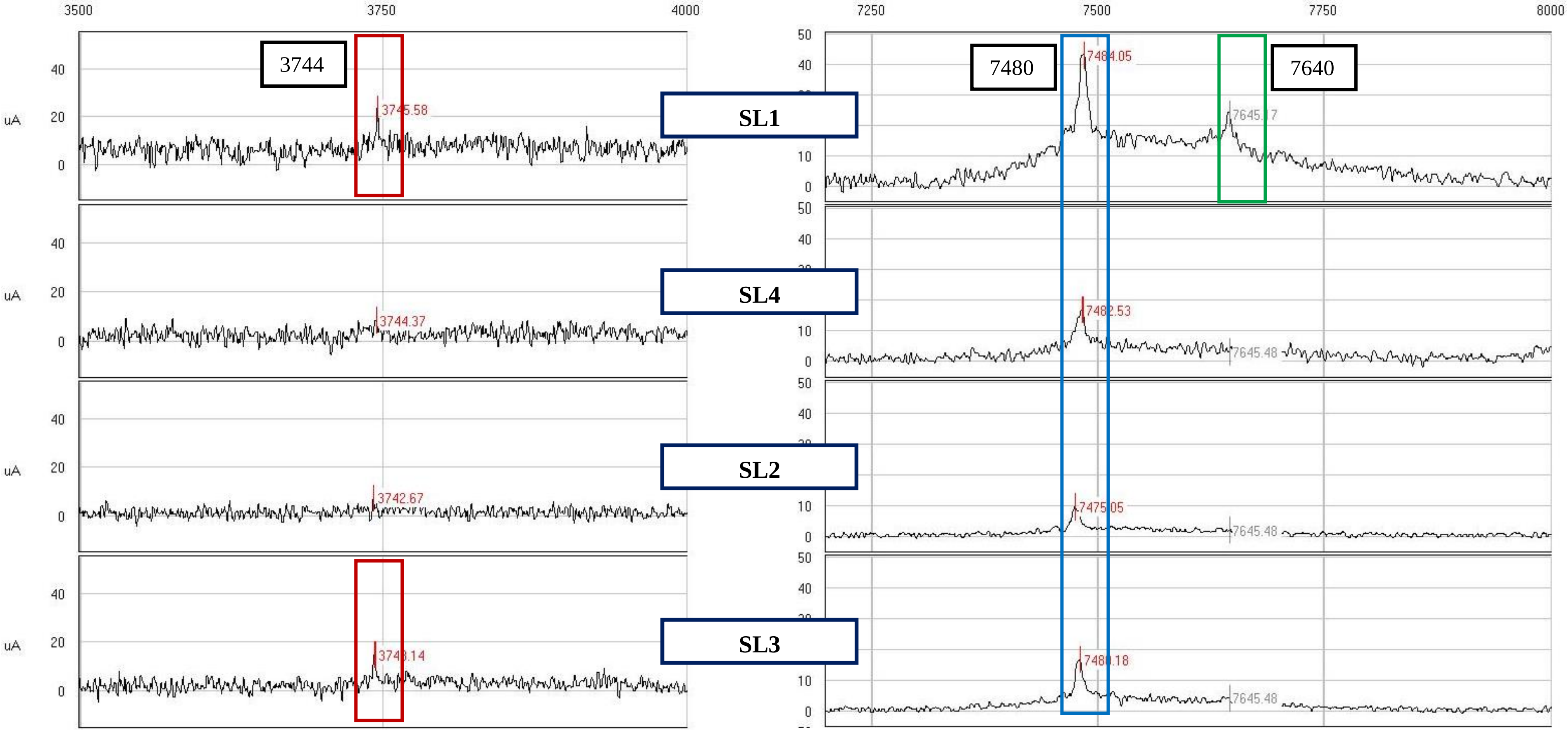


Figure 4.22 Comparison of the peaks in SL SELDI-TOF profiles

Blue box highlighted peak with *m/z* 7480 which is constantly present in all the 4 spectrum profiles. Red box showed peak with *m/z* 3744 present in SL1 and SL3 spectrum profiles, while green box showed peak with *m/z* 7640 which only present in the spectrum of SL1.

Cluster Report

Group	M/Z Avg	M/Z Std	Intensity Avg	Intensity Std	# of peaks	# estimated
Cluster 2	*Condition*	*M /Z*	*7,479.9 P-value*	*0.024*	*ROC area* �	
SL1	7,484.049	0.712	38.47	8.925	3	0
SL4	7,481.739	0.951	19.12	2.492	3	0
SL2	7,477.747	2.656	12.619	2.811	3	0
SL3	7,478.85	1.657	14.245	3.093	3	0

Figure 4.23 Cluster report of peak with *m/z* 7480 in SL SELDI-TOF profiles

Peak clusters were generated for each spectrum profile for SL1, SL2, SL3 and SL4. EDM analysis by comparing the intensities of peak clusters reports a detection of *P*-value at 0.024. Average and standard deviation were written in short as Avg and Std in the report.

Figure 4.24 Cluster plot of peak with *m/z* 7480 in SL SELDI-TOF profiles

Cluster plot revealed that *m/z* 7480 peak intensities for SL1 were highest among the four, followed by SL4 and SL3. SL2 has the lowest peak intensities.

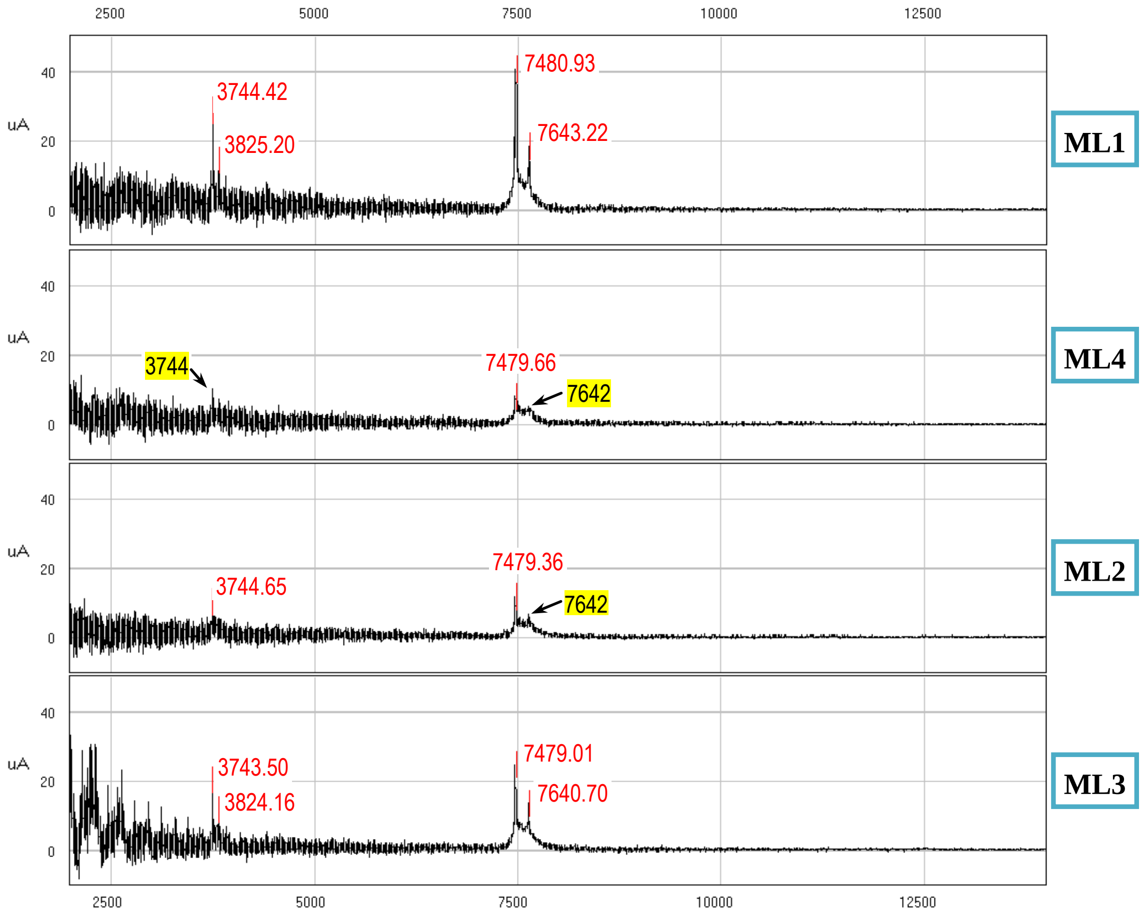


Figure 4.25 SELDI-TOF profiles of ML extracts

This figure showed the representative spectra of ML sample from Method 1 (ML1), Method 4 (ML4), Method 2 (ML2) and Method 3 (ML3). Peaks were automatically detected and normalized to total ion current intensity in 2 – 15 kDa using ProteinChip Data Manager Software 3.5 with the condition of 5.0 S/N valley depths and 5.0 S/N peak heights. The arrow highlighted manually identified peaks that are present below the detection limit.

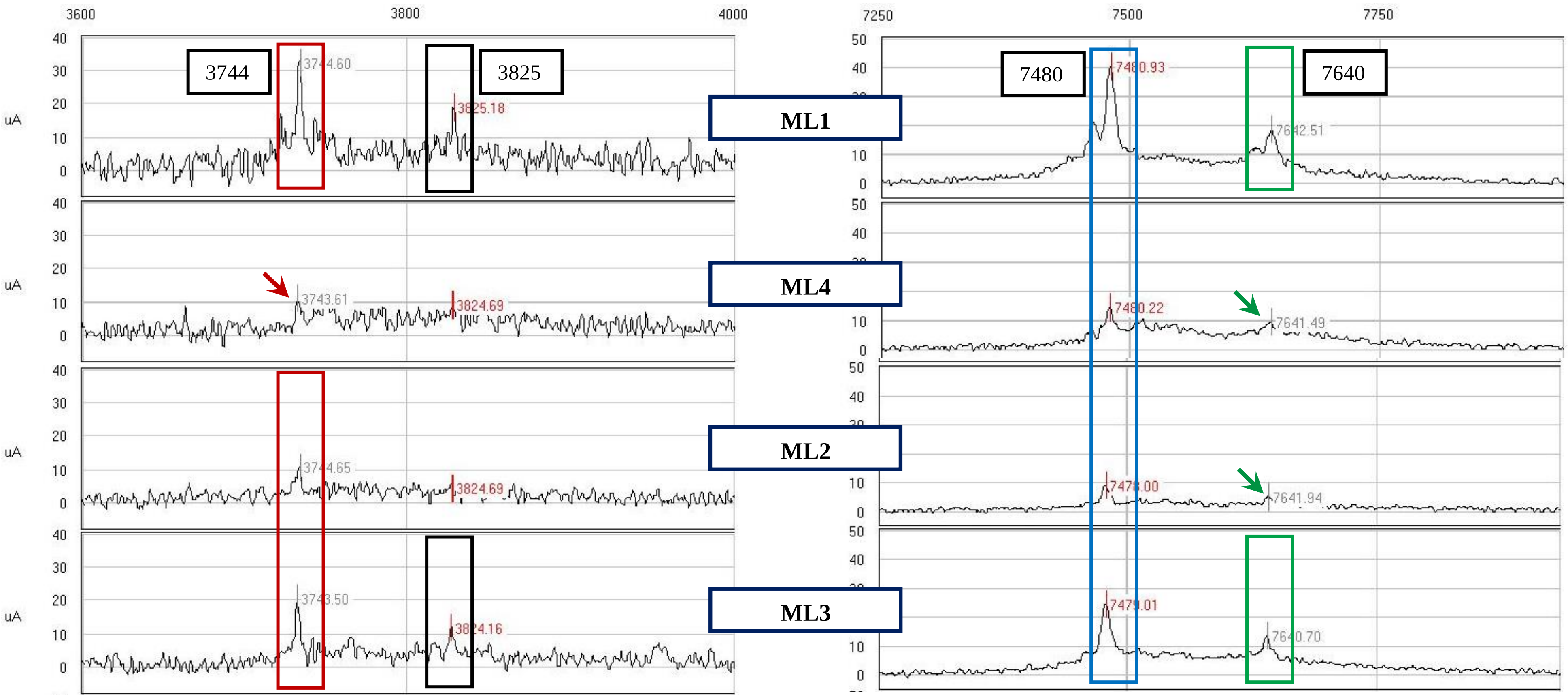


Figure 4.26 Comparison of the peaks in ML SELDI-TOF profiles

Peaks in the four spectrum profiles of ML1, ML4, ML2 and ML3 were compared. Blue box highlighted peak with *m/z* 7480 which is constantly present in all the 4 spectrum profiles. Red, black and green boxes showed detected peaks at *m/z* 3744, 3825 and 7640 respectively, while the arrows indicated observed peaks that are not automatically detected but manually identified.

Cluster Report

Group	M/Z Avg	M/Z Std	Intensity Avg	Intensity Std	# of peaks	# estimated
Cluster 1	*Condition*	*M /Z*	*3,742.7 P-value*	*0.024*	*ROC area* �	
ML1	3,744.448	0.194	29.81	2.382	3	0
ML4	3,743.583	0.851	9.008	4.025	3	1
ML2	3,742.913	1.958	9.66	0.867	3	0
ML3	3,743.564	0.375	17.403	2.62	3	0
Cluster 3	*Condition*	*M /Z*	*7,478.1 P-value*	*0.019*	*ROC area* �	
ML1	7,480.776	0.132	38.016	2.955	3	0
ML4	7,480.779	0.492	12.71	2.115	3	0
ML2	7,477.514	2.133	10.261	1.361	3	0
ML3	7,478.666	0.309	21.543	3.207	3	0
Cluster 4	*Condition*	*M /Z*	*7,639.9 P-value*	*0.016*	*ROC area* �	
ML1	7,642.983	1.053	17.683	1.669	3	0
ML4	7,642.958	1.701	9.329	0.198	3	0
ML2	7,640.134	3.827	5.739	0.765	3	0
ML3	7,640.636	0.496	11.705	1.83	3	0

Figure 4.27 Cluster report of peak with *m/z* 3744, 7480 and 7640 in ML SELDI-TOF profiles

Peak clusters were generated for each spectrum profile for ML1, ML4, ML2 and ML3. EDM analysis by comparing the intensities of peak clusters reports a detection of *P*-value at 0.024, 0.019 and 0.016 for peak cluster with *m/z* 3744, 7480 and 7640 respectively. Average and standard deviation were written in short as Avg and Std in the report.

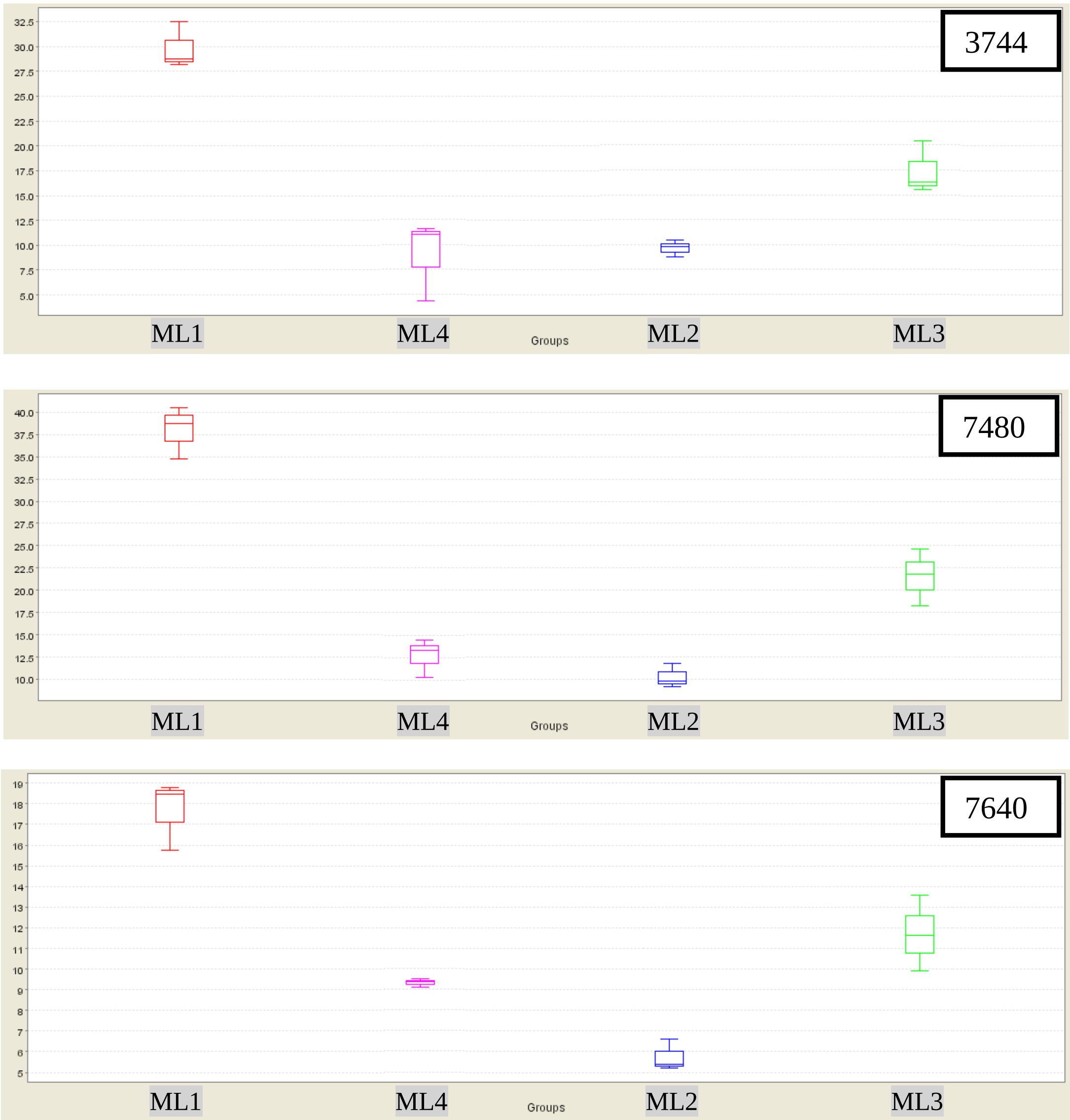


Figure 4.28 Cluster plot of peak with *m/z* 3744, 7480 and 7640 in ML SELDI-TOF profile

Cluster plot revealed that peak with *m/z* 3744, 7480 and 7640 have the highest intensity in ML1 among the four, followed by ML3, ML4 and ML2.

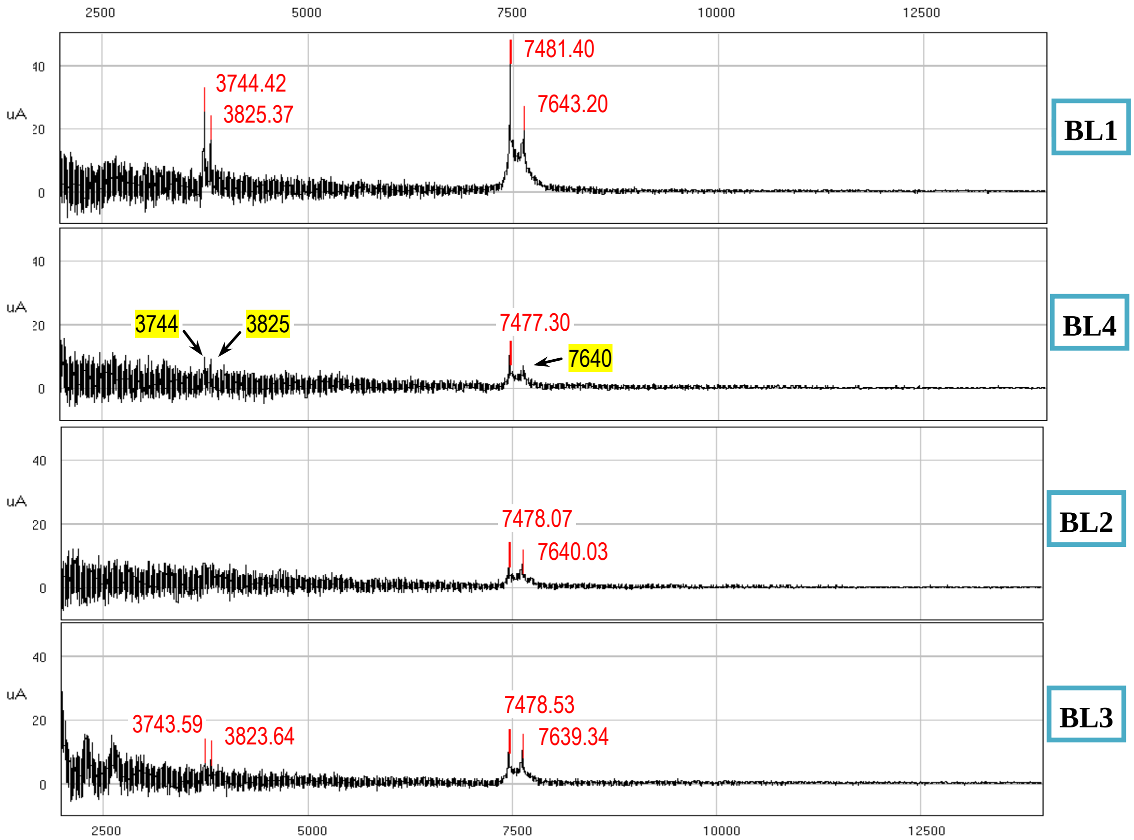


Figure 4.29 SELDI-TOF profiles of BL extracts

This figure showed the representative spectra of BL sample from Method 1 (BL1), Method 4 (BL4), Method 2 (BL2) and Method 3 (BL3). Peaks were automatically detected and normalized to total ion current intensity in 2 – 15 kDa using ProteinChip Data Manager Software 3.5 with the condition of 5.0 S/N valley depths and 5.0 S/N peak heights. The arrow indicated manually identified peaks that are present below the detection limit.

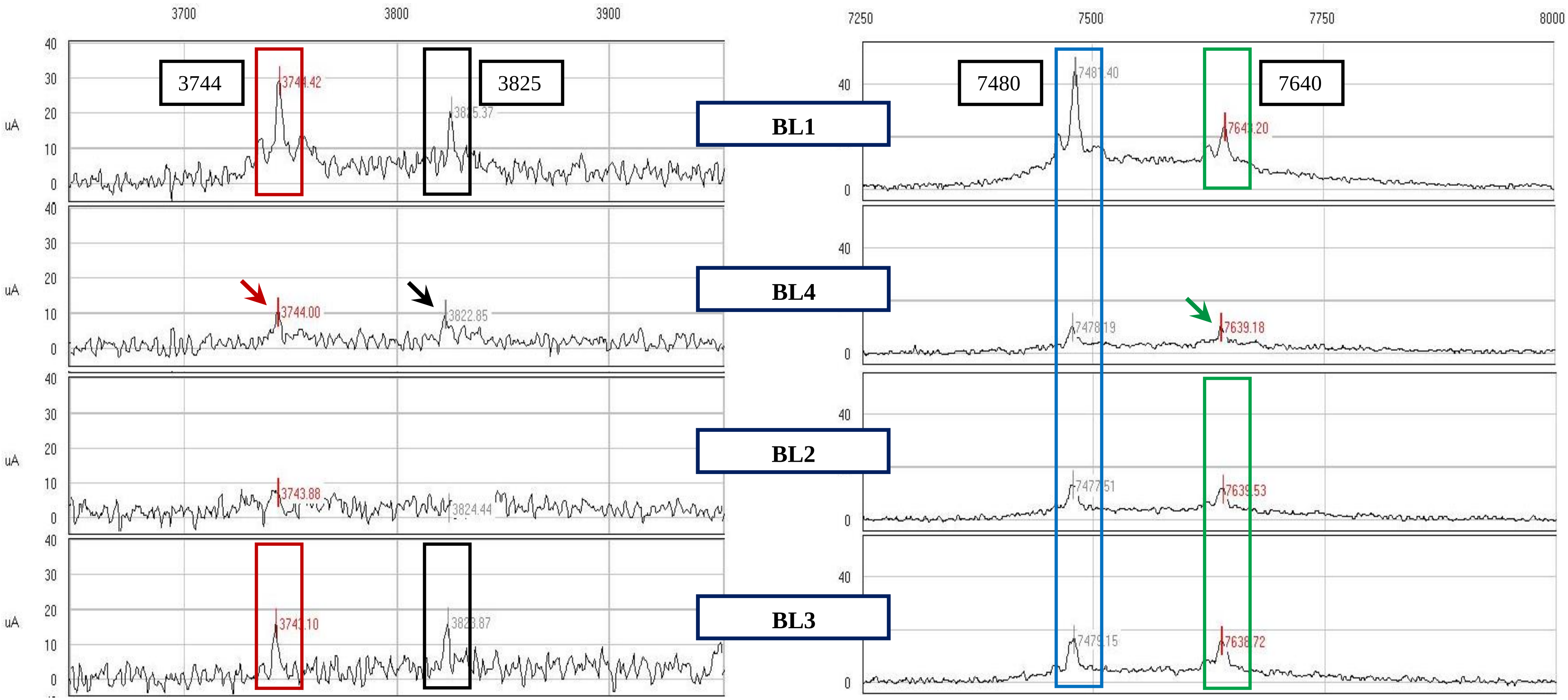


Figure 4.30 Comparison of the peaks in BL SELDI-TOF profiles

Peaks in the four spectrum profiles of BL1, BL4, BL2 and BL3 were compared. Blue box highlighted peak with *m/z* 7480 which is constantly present in all the 4 spectrum profiles. Red, black and green boxes showed the detected peaks at *m/z* 3744, 3825 and 7640 respectively, while the arrows indicated observed peaks that are not automatically detected but manually identified.

Cluster Report

Group		M/Z Avg	M/Z Std	Intensity Avg	Intensity Std	# of peaks	# estimated
Cluster	*3*	*Condition*	*M/Z*	*7,478.5 P-value*	*0.022*	*ROC area*	
BL1		7,481.677	0.403	46.098	6.183	3	0
BL4		7,477.34	2.321	9.324	1.68	3	0
BL2		7,478.112	0.916	10.616	0.419	3	0
BL3		7,478.534	0.892	14.152	2.116	3	0
Cluster	*4*	*Condition*	*M/Z*	*7,639.5 P-value*	*0.016*	*ROC area*	
BL1		7,642.962	0.205	25.607	4.102	3	0
BL4		7,637.598	1.566	6.899	1.164	3	0
BL2		7,639.8	0.935	9.5	1.063	3	0
BL3		7,639.642	0.976	13.062	2.405	3	0

Figure 4.31 Cluster report of peak with *m/z* 7480 and 7640 in BL SELDI-TOF profiles

Peak clusters were generated for each spectrum profile for BL1, BL4, BL2 and BL3. EDM analysis by comparing the intensities of peak clusters reports a detection of *P*-value at 0.022 and 0.016 for peak cluster with *m/z* 7480 and 7640 respectively. Average and standard deviation were written in short as Avg and Std in the report.

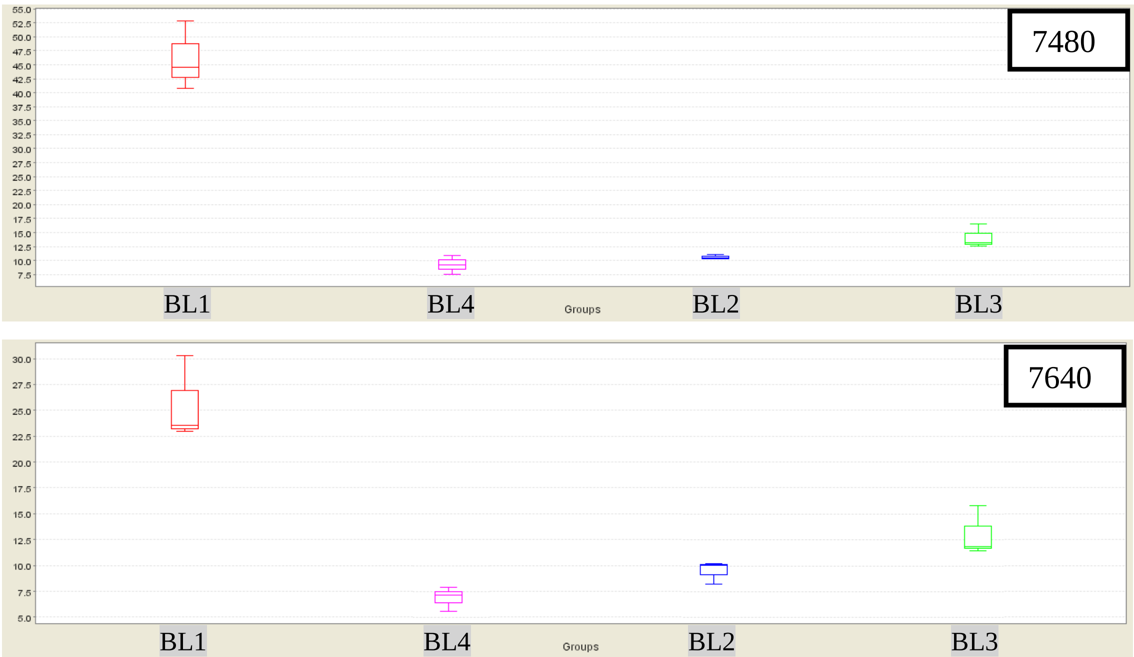


Figure 4.32 Cluster plot of peak with *m/z* 7480 and 7640 in BL SELDI-TOF profiles

Cluster plot revealed that peak intensity of these two peaks were significantly high in BL1, followed by BL3, BL2 and BL4.

Table 4.5 Detected peaks in the SELDI-TOF profiles of SL, ML and BL samples

Sample	Detected Peaks				
SL1	*m/z*	3744.96		7484.04	7645.48
SL4	*m/z*			7481.60	
SL2	*m/z*			7477.71	
SL3	*m/z*	3743.41		7478.59	
ML1	*m/z*	3744.42	3825.20	7480.93	7643.22
ML4	*m/z*	3743.61*****		7479.66	7641.49*****
ML2	*m/z*	3744.65		7479.36	7642.72
ML3	*m/z*	3743.50	3824.16	7479.01	7640.70
BL1	*m/z*	3744.42	3825.37	7481.40	7643.20
BL4	*m/z*	3744.00*****	3822.85*****	7477.30	7639.18*****
BL2	*m/z*			7478.07	7640.03
BL3	*m/z*	3743.59	3823.64	7478.53	7639.34

Peaks were automatically detected with the condition of S/N > 5.0 for valley depths and peak heights. Peaks marked with ***** are manually identified peaks.

4.8.2 Comparison of the SELDI-TOF profiles of the three varieties of *F. deltoidea*

Spectrum profiles of SL1, ML1 and BL1 were used to compare among the varieties as they have the most complete profile. Their representative spectra are shown in Figure 4.33 and comparison of peaks detected in each spectrum profiles was presented in Table 4.6. SELDI-TOF instrument is known to have reading error of approximately 0.2 % for its *m/z* value, thus peak at *m/z* 3745.58 (SL), 3744.51 (ML) and 3744.42 (BL) are considered the same cluster of protein with *m/z* 3744. The same applied to the other 3 peaks. The results showed that peaks with *m/z* 3744, 7480 and 7640 were present in all the three varieties of *F. deltoidea*. However, peak with *m/z* 3825 was only present in the spectrum profiles of ML and BL, it was absent in the spectrum profile of SL.

Table 4.6 Detected peaks in the SELDI-TOF profiles of SL, ML and BL samples obtained from Method 1

Samples	Peaks detected				
SL	*m/z*	3745.58		7484.05	7645.17
ML	*m/z*	3744.51	3825.22	7480.93	7642.51
BL	*m/z*	3744.42	3825.37	7481.40	7643.20
Peak cluster at *m/z*		**3744**	**3825**	**7480**	**7640**

Peaks with *m/z* 3744, 7480 and 7640 were present in the spectrum profiles of all three *Ficus* varieties. Peak with *m/z* 3825 was only present in the spectrum profiles of ML and BL.

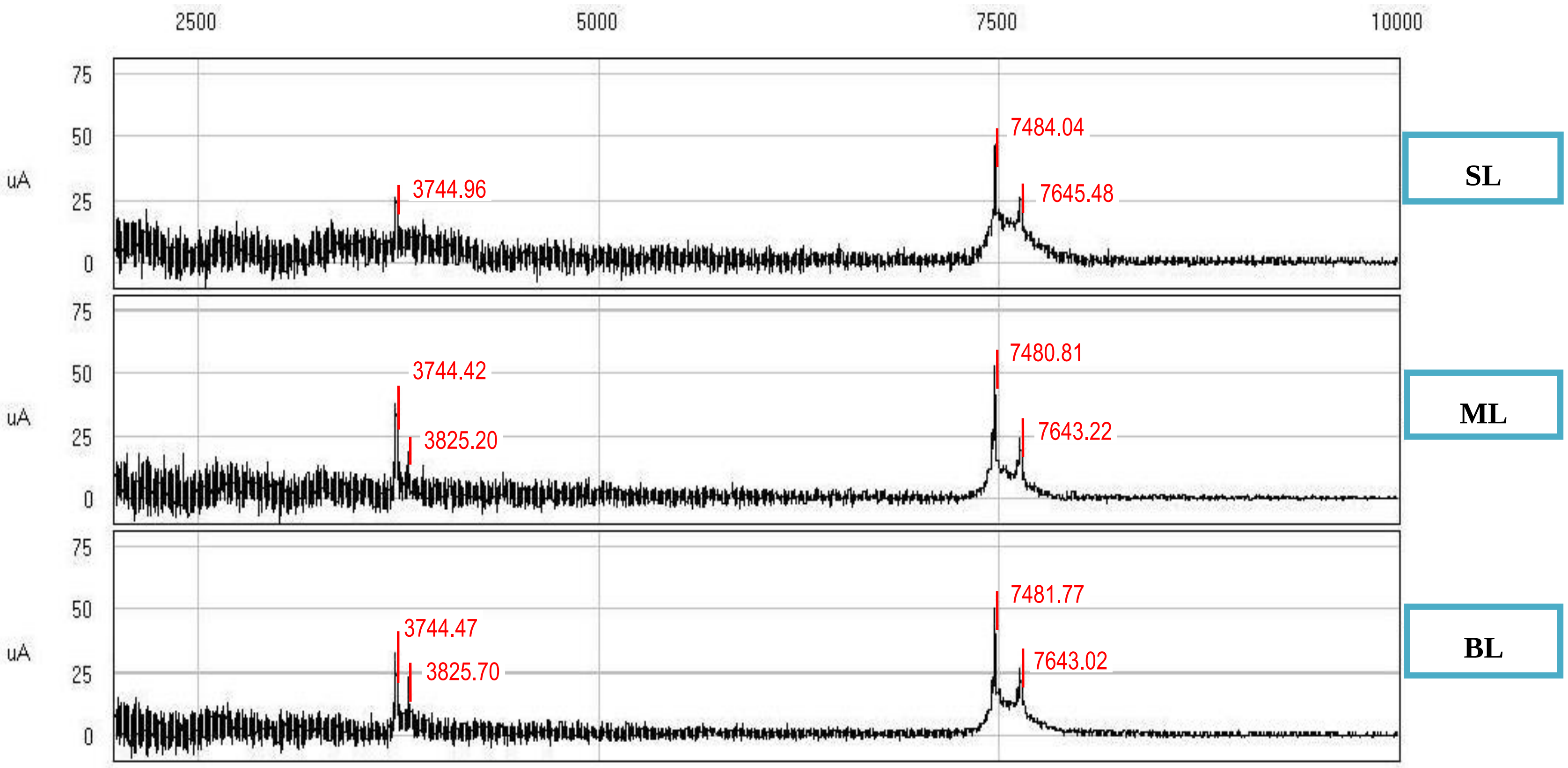


Figure 4.33 Comparison of SELDI-TOF profiles of SL, ML and BL extracts obtained from Method 1

Once spectrum is obtained, peaks were automatically detected and normalized to total ion current intensity in 2 – 15 kDa using ProteinChip Data Manager Software 3.5 with the condition of 5.0 S/N valley depths and 5.0 S/N peak. Peak with *m/z* 3825 is absent in the spectrum profile of SL while other three peaks are present in all the three spectrum profiles.

4.9 Glycoproteins Detection

SDS-PAGE gels were stained with periodic acid-Schiff (PAS) reagent instead of silver stain in order to detect the presence of glycoproteins. Positive and negative controls of glycoprotein were loaded onto the wells on SDS gel. Horseradish peroxidase was used as positive control, whereas the negative control was soybean trypsin inhibitor. The glycoprotein profiles were revealed in Figure 4.34 and 4.35.

A red magenta band was observed at positive control lane indicating the presence of glycoprotein. Magenta bands were observed at the boundary between stacking and separating gel of every sample's lane. Smearing magenta background was also present in sample's lane. Samples obtained from Method 1 (Lane 1, 2 and 3 in Figure 4.34) have darkest smearing background compared to the others. Lanes of samples obtained from Method 4 (Lane 12, 13 and 14 in Figure 4.35) have lesser smearing background compared to the others.

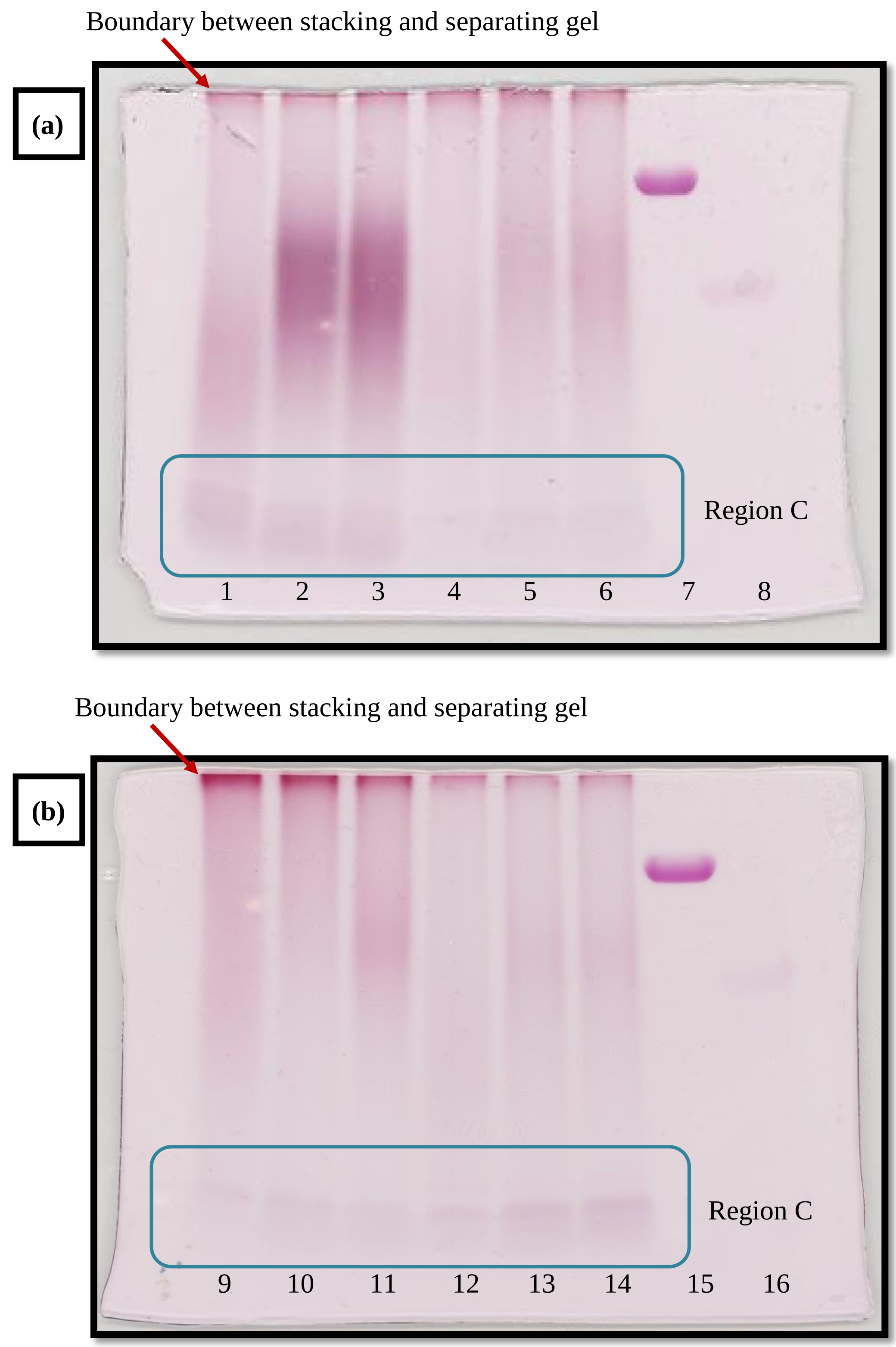


Figure 4.34 Detection of glycoproteins on the SDS-PAGE profiles of *F. deltoidea*

Figure 4.34 Detection of glycoproteins on the SDS-PAGE profiles of *F. deltoidea*

This figure revealed the SDS-PAGE gel stained with periodic acid-Schiff (PAS) reagent to show the presence of glycoprotein. The profiles of *Ficus* samples obtained from Method 1 and Method 2 are presented in (a), while profiles of *Ficus* samples obtained from Method 3 and Method 4 are presented in (b). Positive control (Lane 7 and Lane 15) and negative control (Lane 8 and Lane 16) were presented on the right side of the gel. Red arrow showed the presence of red magenta bands at the boundary between stacking and separating gel. Blue circle indicated Region C in the profile.

Gel (a)	Gel (b)
Lane 1 = SL Method 1;	Lane 9 = SL Method 3
Lane 2 = ML Method 1;	Lane 10 = ML Method 3
Lane 3 = BL Method 1;	Lane 11 = BL Method 3
Lane 4 = SL Method 2;	Lane 12 = SL Method 4
Lane 5 = ML Method 2;	Lane 13 = ML Method 4
Lane 6 = BL Method 2	Lane 14 = BL Method 4

Chapter 5 : Discussions

5.1 Hot Water Extraction

In this study, plant materials were subjected to hot water extraction in a ratio of one part of plant materials to ten parts of water. The reason hot water extraction method was used is to mimic the traditional way of the preparation of *F. deltoidea* for consumption. One of the traditional ways to prepare Mas cotek herb is to boil the leaves, roots or stems in water and consumed as herbal drink (Ong *et al.*, 2011). Decoction of the leaves extracts were freeze-dried for storage purpose before proceed to next step. Freeze-drying is a dehydration process where solvent or water is removed from the material by sublimation. This technique is achieved by freezing the extracts at - 80 °C and then sublime the ice directly to vapour by vacuum. Freeze-dried samples are able to be stored for longer period. Freeze-drying is a preferred method for the preservation of biological samples as it is able to remove water from the sample without disturbing its composition, thus minimizes damage of the internal structure. Moreover, low drying temperature might avoid occurrences of heat induced biochemical reaction and protein denaturation (Bonazzi and Dumoulin, 2011).

5.2 Removal of Polyphenolic Compounds and Tannins

Tannins, polyphenolic compounds, triterpenoids and flavonoids are reported to be present in the extracts of *F.deltoidea* (Abdullah *et al.*, 2009; Hakiman *et al.*, 2012). These phytochemicals can severely affect the proteomics analysis. The presence of phenolics compound may contribute to streaking and generate artifactual spots on 2-D gels (Vâlcu and Schlink, 2006). In this study, polyvinylpolypyrrolidone (PVPP) was used to remove tannins and polyphenolic compounds in the extracts. According to Charmont *et al.* (2005), removal of phenolic compounds by PVPP has improved the recovery and identification of proteins. PVPP is a highly cross-linked form of polyvinylpyrrolidone (PVP), which makes it insoluble in water. It is an effective adsorbent for tannins and polyphenols (Gray, 1978). PVPP interact with polyphenolic compounds by forming stable hydrogen bonds, as insoluble complexes, thus it can be readily removed from the solution by filtration.

Initially when the extracts were re-dissolved in distilled water, the extracts showed a dark brown solution. Phytochemicals that present in the extracts, such as tannins and polyphenols, might contribute to the colour of the extracts. The brown colour of the extracts turns lighter after being treated with PVPP. Also, total phenolic content assay revealed that the phenolic content in the extracts were reduced after PVPP treatment. These results suggested some of these compounds were being removed by PVPP. Filtered extracts of SL, ML and BL were then subjected to protein precipitation methods.

5.3 Comparison of Laemmli Buffer System and Tricine-SDS-PAGE System

In this study, two buffer systems of SDS-PAGE were compared, namely Laemmli buffer system and Tricine-SDS-PAGE system. Laemmli SDS-PAGE system is the most common method used. It utilizes glycine in the running buffer and can resolve proteins in the molecular mass range from over 200 kDa down to 3 kDa. In Tricine-SDS-PAGE system, tricine is used in the running buffer instead of glycine. According to Schägger and Jagow (1987), this system is capable of resolving smaller proteins and gives better resolution of low molecular weight (MW) proteins. Stacking and unstacking of the proteins in the gel plays a major role in band resolution. Glycine used in Laemmli buffer migrates very slow in the acidic stacking gel, leading to stacking of large proteins. For small peptides (< 20 kDa), they tend to form complexes with bulk SDS, thus are not well separated on SDS gel (Schägger and Jagow, 1987). Electrophoretic molibilities of tricine are faster than glycine in a stacking gel at pH between 6.8 and 8.8 which results in the shifting of stacking limit toward low MW range. It thus enabling small peptides separated from the stacked bulk SDS and improves the resolution (Schägger and Jagow, 1987). Lower MW stacking limit enabled proteins to be separated at lower acrylamide concentration.

Comparison of gels resolved by this two buffer system showed both systems were able to produce sharp bands for high MW proteins. Laemmli buffer system is able to resolve low MW proteins (below 6.5 kDa), but with poor resolution. Proteins with MW lower than 6.5 kDa were better resolved in tricine gels. Overall, Tricine-buffer system seems to be a better choice as the sample contains both high and low MW proteins, and low MW protein bands were better resolved in Tricine gel.

5.4 Evaluation of the Four Precipitation Methods in terms of Crude Protein Yield, Protein Content and SDS-PAGE Profile

Water is an excellent and economical solvent, but it has limitation on its selectivity. Generally aqueous extraction can results in the extraction of various polar or ionic compounds that present in the leaves. Since we are focusing on proteins that are present in the leaves extracts, protein precipitation was carried out in order to eliminate components that may interfere with down-stream proteomics analysis and to concentrate proteins from the diluted extracts.

Ammonium sulfate precipitation (Method 1) offers a great advantage in the stabilization of proteins as it is non-denaturing to the protein structure (Scopes, 1994). The principle of this precipitation method is salting out proteins using high concentration of salts, which is ammonium sulfate in this case. Salt ions have greater tendency to be solvated, thereby scavenges water molecules from proteins which leads to the decrease in their solubility and thus precipitate. However, the drawback is any compound with similar solubility behaviour may also precipitate together with the proteins. Dark brown colour of the freeze-dried extract, smearing background in SDS-PAGE profiles and poor resolution of the protein bands, especially in SL sample, suggested other interfering compounds might have been precipitated together with the proteins. Although results showed that extract obtained from this method has the highest purity, but it was also noted that twice as much of proteins was needed for Method 1's samples to visualize the protein profile on SDS-PAGE compared to samples from Method 3 and 4. This shows that the protein content in the extracts was being over estimated.

Similarly, protein content in the extracts obtained from acetone precipitation (Method 2) might also be overestimated as higher amount of protein was needed to visualize the protein profile on SDS-PAGE. Acetone precipitation is easy to perform and has advantage in removing lipids and triglycerides (Lovrien and Matulis, 2001). The principle of this method is to reduce the solvating power of water by increasing the concentration of organic solvent. It gave the highest crude protein yield among the four, but suffers from incomplete solubilisation of the pellet and over estimation of the protein content. This suggested that other interfering compounds were being precipitated together with the proteins.

Extracts obtained from Method 3 and Method 4 have more accurate protein quantification. Method 3 or TCA/acetone precipitation is one of the most commonly reported methods for plant sample. This precipitation method is effective in the elimination of contaminants such as lipids, nucleic acids, polysaccharides and phenol, which cause artifactual spots and affect the separation of proteins during gel electrophoresis (Simpson, 2003; Görg *et al.*, 2004). Its clean up effect is more effective than acetone alone. Moreover, protease activities which can results in artifactual spots and loss of high-mass proteins are inhibited by low pH surrounding (Wang *et al.*, 2008). Light coloured powder extracts and clear background colour in the profile of SDS-PAGE is an indication of interfering compounds being removed. Since acetone is involved in this precipitation, certain protein loss resulting from incomplete solubilisation was unavoidable.

Method 4 is a double protein precipitation method combining ammonium sulfate and acetone/β-mercaptoethanol precipitation. Purity of the extracts are higher compared to extracts obtained from TCA/acetone precipitation, thereby suggesting the combination of two types of precipitation method results in the removal of most interfering compounds. Walliwalagedara *et al.* (2010) reported the combination of TCA/acetone precipitation and phenol treatment prior to 2-D electrophoresis has improved the gel resolution. Double precipitation had advantage of removing different type of interfering compounds as it involves two type of precipitation method, thus enhancing the resolution and revealing more proteins in SDS-PAGE analysis. However, this method is time-consuming and have low crude protein yield due to protein loss in each precipitation method.

The aim of this study is to profile the proteins in *F. detoidea* leaves aqueous extracts, thus extraction method that are able to recover the most proteins is preferred. Method 4 (ammonium sulfate precipitation combined with acetone/β-mercaptoethanol precipitation) gave higher purity, more bands and better resolution on SDS gels, thereby protein extracts of the three plant varieties obtained from this method was selected for further protein profiling of *F. deltoidea*.

5.5 SDS-PAGE and 2-D Gel Electrophoresis Protein Profile of the Three Varieties of *F. deltoidea* Leaves Aqueous Extract

There are some challenges faced in obtaining the 2-D profile. Optimization on certain parameter was carried out. The samples were poorly solubilized in urea rehydration buffer. Solubilization of the proteins prior to 2-D gel electrophoresis is an important step in obtaining good profile (Berkelmen *et al*., 2004). The presence of hydrophobic / membrane proteins might be the reason that results in poor solubilization of the sample. Solubilisation of these proteins can be improved with the use of thiourea (Rabilloud, 1998). Modification of the rehydration buffer was done by including two molar of thiourea. Solublization of the sample was improved after the amendment, however small amount of precipitate still present after centrifugation. Protein aggregation and precipitation occurred during the IEF and poor focusing of the protein spots lead to the difficulties in obtaining reproducible profiles. Also, vertical streaking was constantly present at the left (acidic) side of the profile. It is possible that these streaking were caused by the proteins precipitated in the loading cup during first dimensional isoelectric focusing. The presence of phytochemical in the extracts might be another explanation for these streaking. Besides that, dark bands that present at the boundary between stacking and separating gel of every sample lane were suspected to be glycoproteins. The presence of glycoproteins will be further discussed in Section 5.9.

On top of that, it was observed that protein bands with MW less than 6.5 kDa were missing in all the 2-D proteome of SL, ML and BL. There are a few possibilities which lead to the absence of these proteins. Laemmli buffer system has a limitation in resolving low MW proteins (Schägger, 2006). Low MW proteins are too small to be retain in the gel, thus migrating at the same rate with the frontline, or sometimes faster than the frontline would result in missing protein bands or spots in the proteome. Secondly, the absence of the proteins might due to low intensity or concentration below the detection limit. Those protein bands below 6.5 kDa may consist of a few protein spots with different pI. In 2-D gel electrophoresis, the protein bands were further separated into protein spots with different pI. Therefore, concentrations of the protein bands are further diluted in 2-D proteome and might resulted in their absence. Furthermore, those proteins might have pI lower than 3 or higher than 10, which lead to their loss in 2-D proteome.

SDS-PAGE and 2-D gel electrophoresis analysis revealed that SL leaves aqueous extract contains a bunch of proteins in the range of MW 1.42 kDa up to 101 kDa, with diverse abundancy. They can be categorized into three groups based on their MW as shown in SDS-PAGE profile. The group of protein bands in Region A on SDS-PAGE profile (Figure 4.12) were further resolved in 2-D profile, which resulted in 33 spots in Region X on 2-D profile (Figure 4.13). Six protein bands in Region B on SDS-PAGE profile (Figure 4.12) were presented in Region Y on 2-D profile (Figure 4.13). Comparing Region B (Figure 4.12) and Region Y (Figure 4.17) showed that Band 33.66 ± 0.44 kDa was further separated into spot Y1 and Y2, Band 24.95 ± 0.57 kDa was further separated into spot Y3 and Y4, and Band 22.93 ± 0.18 kDa was resolved into spot Y5 and Y6. Spot Y7 was Band 15.89 ± 0.46 kDa. No band was observed in between Band 15.89 ± 0.46 kD and Band 13.56 ± 0.50 kDa on SDS-PAGE profile of SL. However, a fade spot was observed at Y8 in the SL's 2-D profile. Since higher amount of proteins were loaded into 2-DE gel, intensity of low abundance proteins were enhanced. This might be the reason that spot Y8 was observed in 2-D profile but not in SDS-PAGE profile. Band 13.56 ± 0.50 kDa was resolved into spot Y9 and Y10, whereas Band 12.56 ± 0.14 kDa was resolved into Y11 and Y12. Due to the limitation of Laemmli buffer gel in resolving low MW proteins, Band 7.48 ± 0.17 kDa, 3.82 ± 0.47 kDa, 2.48 ± 0.20 kDa and 1.91 ± 0.04 kDa were not resolved in 2-D profile of SL. Besides that, most of the proteins were low abundance, as we can see most of the spots were fade.

ML leaves aqueous extract contains two groups of proteins. First group of proteins (in Region A on SDS-PAGE profile) lies within MW 53 kDa – 78 kDa, while the other group of proteins (in Region C on SDS-PAGE profile) lies between 1.42 kDa – 6.51 kDa (as shown in Figure 4.12). Proteins in Region A were further resolved in 2-D gel at Region X (Figure 4.14) which resulted in 24 spots detected. Similarly, BL leaves aqueous extract contains two groups of proteins (as shown in Figure 4.12). First group of proteins (in Region A on SDS-PAGE profile) lies in the range between 53 kDa – 78 kDa. Second group of proteins (in Region C on SDS-PAGE profile) lies within 1.42 kDa – 6.51 kDa. Looking into the SDS-PAGE profile (Figure 4.12) and 2-D profile of BL (Figure 4.15), Group A proteins on SDS-PAGE profile were presented in Region X on 2-D profile. Nine spots were detected in this region (shown in Figure 4.19). Second group of proteins of ML and BL which lies between 1.42 kDa – 6.51 kDa were not resolved in their 2-D profile due to the MW limitation of 2-D gel electrophoresis. Certain spots detected were very fade, which indicates low abundance of the proteins. This suggested that most of the proteins were low in abundance.

5.6 Comparison of the Protein Profiles among the Three Varieties of *F. deltoidea*

Among the three varieties (SL, ML and BL) of *F. deltoidea*, SL has the most number of spots detected, followed by ML and BL. SL profile has a distinct SDS-PAGE and 2-D pattern from ML and BL profiles. SL profile contains a group of proteins at MW region between 6 – 28 kDa, which are not present in the profiles of ML and BL. Since they are only present on SL profile, these proteins are characteristic proteins for SL. ML and BL have similar SDS-PAGE profile. The four protein bands in Region C on ML profile have similar MW with the four protein bands in Region C of BL profile, thereby they might be the same proteins. All the three varieties of *F. deltoidea* contain a group of proteins which lays within MW range of 53 kDa – 78 kDa (Region A) in SDS-PAGE profile.

In 2-D profile, this group of proteins showed similar pattern among the three varieties. Seemingly most of the protein spots were present in a series of lines. For example at L3 (Figure 4.16), there are 14 spots present in a horizontal line with same molecular weight but different pI value. Potentially, these spots might be proteins with heterogeneous phosphorylation. Heterogeneous phosphorylation of a protein is known to gives a series of spots with same molecular weight but different pI values (Seo and Lee, 2003; Larsen and Roepstorff, 2000). However, further investigation is required as more evident is needed to determine the type of modification on two-dimensional gels (Halligan *et al.*, 2004). The same goes for L1, X2, and X3. However, there are a number of protein spots were missing in ML and BL 2-D profiles. Looking into Region X on SL, ML and BL 2-D profiles (Figure 4.16, 4.18 and 4.19), L1 is only observed in SL profile and L3 is missing in BL profile. This L1 protein could be unique to SL. L3 proteins could be common among SL and ML, the absence of them in BL might help to differentiate BL from ML. At L2, the spot pattern is slightly different among each other with eight spots in SL profile, followed by three and two spots in ML and BL profile.

Protein spots in L2 might represent a fingerprint for each of the three plant variety. The number and position of the spots might provide additional criteria for authentication purposes. Overall, SL profile contains 33 protein spots in this region, whereas ML and BL profiles have 24 and nine spots detected respectively.

5.7 MALDI-TOF-TOF Identified Proteins

MALDI analyses of all the spots were difficult due to insufficient amount of protein and challenges in getting reproducible gels. Hence, only certain spots with higher intensity were selected to proceed with MALDI-TOF-TOF analysis. Ten protein spots were sent for MALDI analysis.

In Region X, it is noted that the gel MW of X4 is significantly lower than the predicted weight. The gel spot might represent a protein fragment of the identified protein. The proteins might have been degraded into smaller fragments during the high heat extraction or break down by proteases. On the other hand, X1 and X2 have higher gel MW compared to predicted weight. This may indicate the presence of protein modification such as glycosylation and phosphorylation. Especially in glycosylation, the protein covalently attached to carbohydrates moiety which leads to conformational change in the protein and increase in size, thus leading to abnormal behaviour on the gel (Wu *et al.*, 2013). These modifications might occur during the high heat extraction step. All different components were present during aqueous extraction, and high heat encourages the modification to occur. Same observation in Region Y, all protein spots identified in this region are significantly lower than the predicted weight. On top of that, Y3, Y4, Y5 and Y6 are identified as same protein. They might be fragments of protein which resulted from proteolytic activity of high heat extraction.

Three common proteins for the three *Ficus* plant varieties that being identified are photosystem II reaction center protein I (X1), thioredoxin M-type (X2) and glutamyl-tRNA reductase 2 (X3). Photosystem II reaction center protein I is a protein that involved in photosynthesis. It plays a role in controlling the interaction of photosystem II cores with the light-harvesting antenna. Both thioredoxin M-type and glutamyl-tRNA reductase 2 are chloroplast precursors. Thioredoxin M-type involved in various redox reactions through the reversible oxidation of the active center dithiol to a disulfide. There are two forms of thioredoxin; F and M form. The M form of thioredoxin is known to activate NADP-malate dehydrogenase, and deactivates glucose-6-phosphate dehydrogenase (Buchanan *et al.*, 1989). On the other hand, glutamyl-tRNA reductase that belongs to the glutamyl-tRNA reductase family is part of the porphyrin biosynthesis. It catalyzes the NADPH-dependent reduction of glutamyl-tRNA (Glu) to glutamate 1-semialdehyde (GSA) (Schauer *et al.*, 2002), which then converted into 5-aminolevulinate by 5-aminolevulinate synthase. It plays a vital role in chlorophyll biosynthesis. The protein spot (X4) which is common in SL and ML proteome is being identified as sucrose synthase or sucrose-UDP glucosyltransferase. This is a sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways.

Among those SL unique proteins selected for identification, Y3, Y4, Y5 and Y6 were identified as ribulose-1,5-biphosphate carboxylase/oxygenase large subunit. These four protein spots might be fragments of ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) large subunit. This enzyme located in chloroplast and plays an important role in photosynthesis. It catalyzes the conversion of atmospheric carbon dioxide to energy-rich molecules through carboxylation of ribulose-1,5-biphosphate and also catalyzes oxygenation reaction of oxygen and ribulose-1,5-biphosphate which lead to photorespiration. The active site of the enzyme, which is responsible for the carbon fixation, is located in the large subunit (Kellogg and Juliano, 1997). On the other hand, both Y10 and Y12 were identified as a predicted protein of ATP synthase CF-1 alpha subunit. This protein is part of the ATP synthase and is involve in the synthesis of ATP from ADP in the presence of a proton gradient across the membrane (Nakamoto *et al.*, 2008).

Since these proteins play an important role in plant photosynthesis, they should be present in all the extracts. However, they might differ in abundance which resulted in their absence in the protein profile of ML and BL. Visualization of wide dynamic range of proteins on 2-DE gel is a challenge. There is competition between high and low abundance protein in moving to the gel. High abundance proteins dominate in 2-DE gel as they dominate the protein content, hence resulting in low sensitivity of low abundance proteins. RuBisCO and ATP synthase CF-1 alpha subunit might present in higher abundance in SL extracts compared to ML and BL, therefore they are observed and detected in SL proteome. On the other hand, ML and BL might have lower abundance of RuBisCO and ATP synthase in their extracts which resulted in the absence of the two proteins. The presence of these two proteins in SL proteome might provide added fingerprint evidence in differentiating the variety from the others.

5.8 Low MW Hydrophobic Proteins Profiling of *F. deltoidea* Leaves Aqueous Extracts by SELDI-TOF

ProteinChip H50 was used in this experiment. Surface of ProteinChip H50 consists of methylene chain that has binding characteristic similar to C6 to C12 alkyl chromatographic resin. It binds to proteins through hydrophobic interaction or reverse-phase chromatography. Profiling of *F. deltoidea* sample on ProteinChip H50 revealed low MW hydrophobic proteins in the leaves extracts.

5.8.1 Comparison of SELDI-TOF Spectrum Profile of SL, ML and BL from Different Precipitation Methods

The analysis showed samples obtained from different precipitation gave distinctly different SELDI spectrum profile. There were a total of three peaks detected in SL sample and they were peak with *m/z* value 3744, 7480 and 7640. Since *m/z* 3744 is half the value of *m/z* 7480, peak with *m/z* 3744 might be doubly charged peak of *m/z* 7480. SL samples obtained from Method 1 have the most complete spectrum profile among the four as it has all the three peaks observed and detected. Profiles of other samples (SL2, SL3 and SL4) have certain peak missing in their profile. For example, peak with *m/z* 3744 was absent in the spectrum profiles of SL4 and SL2 and peak with *m/z* 7640 was missing in the spectrum profiles of SL4, SL2 and SL3. Protein peak *m/z* 7640 might be washed off during protein precipitation. The absence of peak with *m/z* 3744 (which is the doubly charge of *m/z* 7480) might due to conformational change of the protein with *m/z* 7480 during precipitation.

ML sample contains a total of four protein peaks in their spectrum and they were peak with *m/z* 3744, 3825, 7480 and 7640. The value *m/z* 3744 and 3825 are half the values of *m/z* 7480 and 7640 respectively, thus the former might be the doubly charged of the latter. ML1 and ML3 have similar spectrum profile where all the four peaks are present in their spectrum. ML2 and ML4 have a missing peak at *m/z* 3825 in their spectrum profiles. Conformational change of the protein with *m/z* 7640 which results from the precipitation might have lead to the absence of peak with *m/z* 3825. It was noted that samples which undergo acetone precipitation (ML4, ML2 and ML3) have shown significant loss in their peak intensities.

Similar to ML sample, BL sample contains a total of four protein peaks their spectrum and they were peak with *m/z* 3744, 3825, 7480 and 7640. It is suggested that *m/z* 3744 and 3825 are doubly charged peak of the singly charged protein *with m/z* 7480 and 7640 respectively, as they (3744 and 3825) are half the *m/z* value of 7480 and 7640. BL1, BL3, BL4 have all the four peaks present in their spectrum profiles, with significantly higher peak intensities in BL1 compared to the others. Although BL4 have all the four peaks observed in their profiles, but low quality peaks with *m/z* 3744, 3825 and 7640 were not detected. Meanwhile, BL2 has two missing peak at *m/z* 3744 and 3825. It is suggested that proteins with *m/z* 7480 and 7640 had undergone some conformational changes during the precipitation that lead to the absence of their doubly charged protein peaks (*m/z* 3744 and 3825).

Generally, samples obtained from Method 1 have the most protein peaks comparing to the others. Also, samples obtained from Method 1 showed better peak resolution and higher intensity. Comparing the spectrum profile of samples obtained from Method 1 and 4 revealed that additional washing step with acetone in Method 4 might have enhanced the sensitivity of the low abundance proteins that had been hindered by interference compound or other high abundance proteins in the extract, which resulted in the present of the humps at *m/z* 4000 – 5800, 6799, 8009, 10719 and 13614 in the spectrum of SL4. At the same time, acetone precipitation had also lead to the lost of certain peaks, such as the absence of peak with *m/z* 7640 in SL4. Moreover, their intensities were significantly reduced and lead to low peak quality. For example, peaks at *m/z* 3740 and 7642 in ML4 and peaks with *m/z* 3740, 3825 and 7640 in BL4 were failed to be detected. Since SL1, ML1 and BL1 samples have the most complete profiles among the four, they were selected for the comparison of different plant varieties SELDI profiles.

5.8.2 Comparison of SL, ML and BL SELDI-TOF Protein Profile

Profiles of the three varieties of *F. deltoidea* from Method 1 were used to compare among the varieties as they have more complete profile. Protein peak with *m/z* 3744, 7480 and 7640 are common protein peak that present in spectrum profiles of the three *Ficus* plant variety aqueous extracts. ML1 and BL1 share a unique protein peak with *m/z* value of 3825, which was not present in the SL sample. The presence of protein peak with *m/z* value of 3825 (which is the doubly charged peak of protein with *m/z* 7640) is unique to ML and BL SELDI spectrum profile, which differentiate them from SL. - reconstruct

5.9 Glycoproteins Detection on SDS Gels

Distinct bands that present at the boundary between stacking and separating gel indicated the presence of glycoproteins in the extracts. Large glycoproteins were restrained from entering the acrylamide gel due to their large size. They were hold back at the edge of separating gel. This explained the presence of dark bands at the boundary between stacking and separating gel of every sample lane in SDS gel stained with silver stain. Smearing background was also observed in glycoprotein profile. Comparison between the glycoprotein profiles (Figure 4.34) and SDS-PAGE protein profiles stained with silver staining (Figure 4.12) revealed that the presence of glycoproteins had partly contributed to the smearing background in SDS-PAGE protein profiles. Glycoprotein, which consists of a protein with a glycan chain attached to it may have numerous different glycoforms depending on the glycan structure. The heterogeneity of the glycoform may results in smearing appearance of glycoproteins in SDS gel (Kennedy and Harnett, 2001). Generally, samples precipitated from Method 1 have more smearing background compared to the others, whereas Method 2, 3 and 4 which involve acetone have much lesser background smearing. Acetone precipitation has been effective in removing most of the glycoproteins that caused smearing background in the sample. Since a faint band was observed in the negative control's lane, faint bands at Region C might be false positive results or just an artifact, they are not glycoproteins.

These glycoproteins might be originated from *F. deltoidea* leaves itself, or formed through glycation during hot water extraction. During hot water extraction, various compounds could be extracted by water, especially water soluble compounds. Since sugar is hydrophilic and glycation is favorable in high temperature (Uribarri *et al.*, 2010), it was possible that they were extracted and reacted with proteins in the aqueous extracts during hot water extraction. Moreover, glycation could also occur during the drying (Oliver *et al.*, 2005) and lyophilisation (freeze-drying) process (Zheng *et al.*, 2006).

Chapter 6 : Conclusion

Decoction of *F. deltoidea* leaves contains various types of compounds. In order to profile the proteins/peptides in the decoction, removal of compounds such as phytochemicals, polysaccharides and lipids is essential as they cause severe disturbance in the proteomic analysis. Protein precipitation prior to proteomic analysis could remove interfering compound from the proteins. Among the four precipitation methods evaluated, double precipitation of ammonium sulfate precipitation followed by acetone/β-mercaptoethanol precipitation showed a more efficient removal of these compounds and gave higher purity of protein extracts. SL decoction contains a bunch of proteins that can be categorized into three groups according to their MW; they are in the range of MW 53 – 78 kDa, 6.5 – 26.6 kDa and 1.42 - 6.5 kDa. 2-D protein profile of SL revealed the presence of 33 and 12 protein spots in the group of MW 53 – 78 kDa and 1.42 - 6.5 kDa respectively. Decoction of ML and BL contain 2 groups of protein lies within MW 53 – 78 kDa and 1.42 – 6.51 kDa. ML and BL have similar protein profile. However, ML protein profile contains 24 protein spots in the group of MW 53 – 78 kDa, whereas there are only nine protein spots present in this group on BL protein profile. 12 proteins within MW range of 6.5 – 26.6 kDa on SL protein profile are characteristic proteins for SL. Out of the 12 proteins, six spots were being identified. Four out of the six are being identified as RuBisCO large subunit and the remaining two are identified as ATP synthase CF-1 alpha subunit. Decoction of *F. deltoidea* leaves also contains some low MW hydrophobic proteins and showed the presence of glycoprotein which lead to smearing background. SELDI profiling also showed the similarity in ML and BL spectrum profile where four signal peaks are obtained at *m/z* 3744, 3825, 7480 and 7640. As for SL spectrum profile, missing signal peak with *m/z* 3825 had differentiated SL from ML and BL. Differences among these protein profiles can be used for future studies on this plant and also for authentication purposes.

Chapter 7 : References

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Chapter 8 : Appendix

Table 8.1 Crude extract yield of SL, ML and BL

Plant Sample	Crude Extract Yield (g / 100 g plant material)
SL	16.83 ± 0.85
ML	12.13 ± 0.68
BL	12.50 ± 1.15

Table 8.2 Absorbance values of different BSA concentration

BSA Concentration, µl / mL	Average blank-corrected absorbance value at 562 nm
25	0.0253
125	0.1457
250	0.2970
500	0.5830
750	0.8073
1000	1.0440
1500	1.4913
2000	1.9380

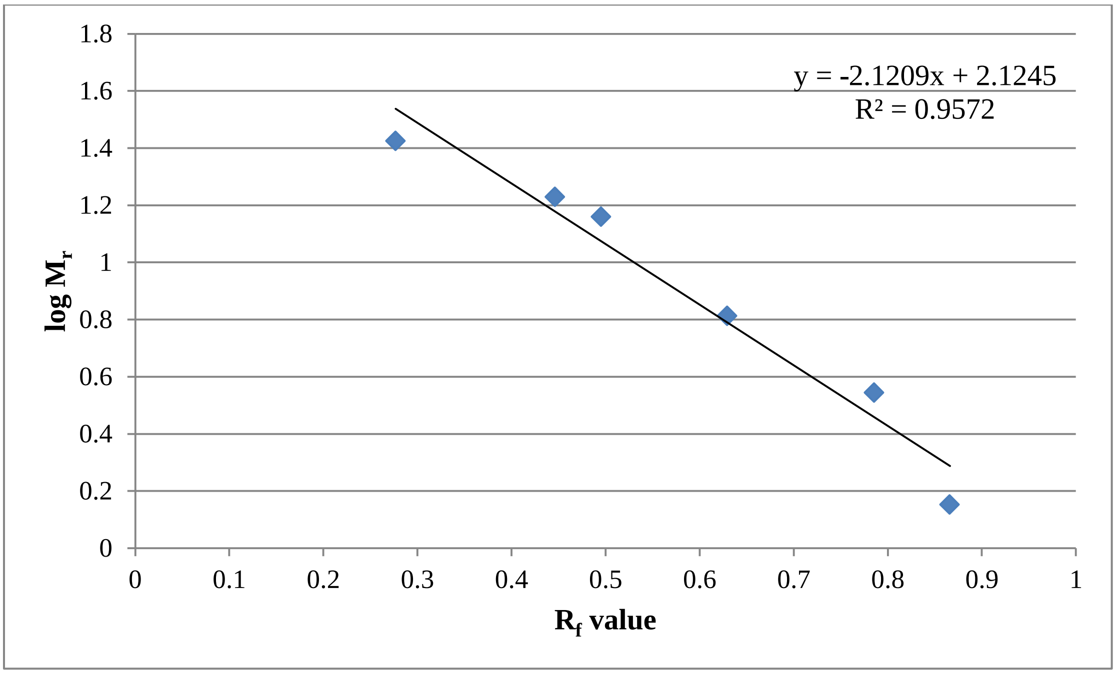

Figure 8.1 Calibration curve for MW determination

Log of known MW standard protein were plotted against their respective R_f values to obtained a calibration curve for MW estimation of protein bands on SDS-PAGE gel. Equation obtained from the calibration curve is y = -2.1209 + 2.1245 with R^2 0.9572.